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**Characterisation and host-parasite
interaction of the piscine diplomonad
*Spironucleus salmonis***

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diplomonad *Spirotrunculus verrucosus***

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I would like to dedicate this thesis to my loving parents....

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DECLARATION

This is to certify that:

- (i) the thesis comprises only my original work towards the PhD
except where indicated,
- (ii) due acknowledgement has been made in the text to all other
material used

Mohammad Reza Saghari Fard

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Peer reviewed papers

1. **Saghari Fard MR**, Jørgensen A, Sterud E, Bleiss W, Poynton SL (2007). Ultrastructure and molecular diagnosis of *Spironucleus salmonis* (Diplomonadida) from rainbow trout *Onchorhynchus mykiss* in Germany. *Diseases of Aquatic Organisms* 75: 37-50.
2. **Saghari Fard MR**, Weisheit C, Poynton SL (2007). Intestinal pH profile in rainbow trout *Onchorhynchus mykiss* and microhabitat preference of the flagellate *Spironucleus salmonis* (Diplomonadida). *Diseases of Aquatic Organisms* 76: 241-249.

Non-peer reviewed paper

3. Poynton SL, **Saghari Fard MR**, Bleiss W, Jørgensen A, Weisheit C, Meinelt T, Rennert B, Cheng J, Kirschbaum F, Knopf K (2007). Towards improved management of infection in aquaculture: strategies arising from the host-parasite interactions in rainbow trout *Oncorhynchus mykiss* and the pathogenic flagellate *Spironucleus salmonis*. *IGB Annual Report 2006 "Jahresforschungsbericht 2006"* 24: 131-140.

Manuscripts in preparation

4. Poynton SL, **Saghari Fard MR**, Huso DL. Is enteropathy in juvenile rainbow trout *Oncorhynchus mykiss* associated with infection by *Spironucleus salmonis* (Diplomonadida)?
5. Poynton SL, **Saghari Fard MR**. Attachment, colony formation, and encystment in *Spironucleus salmonis* (Diplomonadida).
6. Poynton SL, Cheng J, **Saghari Fard MR**, Knopf K. Testing susceptibility to diplomonad infection; development of a new plasma incubation test.

CONTRIBUTIONS TO PAPERS

Published

1. **Ultrastructural and molecular diagnosis of *S. salmonis*:** I examined the fish, made wet preparation of diplomonads, and fixed them for electron microscopy and sequencing. I worked with my co-authors in interpretation of TEM and SEM micrographs. I wrote most aspects of the paper concerned with ultrastructural identification of diplomonads, and I arranged the figures and figure legends. As senior and corresponding author, I was responsible for communicating with the journal editorial for submitting the manuscript, for coordinating the co-authors response to the reviewer's comments, and for communicating with the copy editor for correcting the page proofs.
2. **Intestinal pH and the microhabitat preference of *S. salmonis*:** I examined most of the fish, and recorded occurrence and density of diplomonads, and intestinal pH. I interpreted the results, and wrote most of the manuscript; the tables and figures were prepared, and the statistical analysis conducted, in cooperation with my co-author, Claudia Weisheit. I assisted with written responses to the extensive reviewer's comments, and I re-analysed all of the data as requested, and assisted with rewriting the manuscript. I was responsible for contact with the journal editor and copy editor for all steps of the publication process (as described for paper 1).

In preparation

3. **Pathology associated with *S. salmonis*:** I examined fish, and recorded occurrence and density of diplomonads. I fixed intestine and liver in formalin, and embedded in paraffin. I sectioned and stained the tissues with H&E, PAS, and AB. From my co-authors, I learned interpretation of histopathology, and I then read all of the slides, and took the photographs. I helped my co-authors with writing the manuscript, and I helped prepare the figures and figure legends. I helped Dr. Poynton prepare the manuscript for submission.
4. **Multi-functionality of flagella in *S. salmonis*:** I examined the fish for diplomonads. I ordered the culture materials, and prepared media in lab. I established primary cultures of diplomonads, monitored them, made secondary cultures, and upon observing attachment and encystment, I fixed diplomonads for light microscopy photography, and electron microscopy. I took digital light microscopy photographs of trophozoites and cysts colonies. I arranged the figures, and prepared the figure legends.
5. ***In vitro* plasma incubation test – host susceptibility to *S. salmonis*:** I prepared most of the cultures, and incubated diplomonads into the cultures. I prepared some of the plasma from fish, I developed protocol by determining the following parameters: density of trophozoites in the well, plasma dilution solution, serial dilution, time check points, lysis and cytotoxicity, and number of controls and replications. I performed the modified protocol on cultured *S. salmonis* in different plasma concentrations of rainbow trout carp, and sturgeon.

CONFERENCE PRESENTATIONS

2008

- ✚ Poynton SL*, Huso DL, **Saghari Fard MR** "Unravelling the mystery of the pathogenesis of *Spironucleus* (Diplomonadida), with a quantitative study of *S. salmonis* enteropathy in rainbow trout *Oncorhynchus mykiss*", invited "State of the Art" presentation at 39th Annual Conference of International Association for Aquatic Animal Medicine (IAAAM), Rome, Italy, May 2008

2007

- ✚ **Saghari Fard MR***, Poynton SL "Characterisation, encystment, and microhabitat preference of *Spironucleus salmonis* (a diplomonad flagellate) in *Onchoryhnchus mykiss* (rainbow trout)" at 7th International Symposium on Fish Parasites, (ISFP) Viterbo, Italy, September 2007
- ✚ Poynton SL*, **Saghari Fard MR** "Review of *Spironucleus* spp. (Diplomonadida): improved characterisation, and new insight into parasite-host interactions and pathology" at 7th International Symposium on Fish Parasites (ISFP), Viterbo, Italy, September 2007
- ✚ **Saghari Fard MR*¹**, Weisheit C, Poynton SL "Dose pH affect microhabitat preference of the pathogenic diplomonad *Spironucleus salmonis* in the intestine of rainbow trout *Onchoryhnchus mykiss*?" at 38th Annual Conference of International Association for Aquatic Animal Medicine (IAAAM), Orlando, Florida, May 2007
- ✚ Poynton SL*, Sterud E, Jørgensen A, **Saghari Fard MR** "A review of piscine diplomonads: update on *Spironucleus salmonis* et al." at 38th Annual Conference of International Association for Aquatic Animal Medicine (IAAAM), Orlando, Florida, May 2007

2006

- ✚ **Saghari Fard MR^{*1,2}**, Poynton SL “Attachment, colony formation and encystment in *Spironucleus salmonis* (Diplomonadidae)” at 37th Annual Conference of International Association for Aquatic Animal Medicine (IAAAM), Nassau, Bahamas, May 2006
- ✚ Poynton SL*, Cheng J, **Saghari Fard MR**, Knopf K “Testing susceptibility to diplomonad infection; development of a new plasma incubation test” at 37th Annual Conference of International Association for Aquatic Animal Medicine (IAAAM), Nassau, Bahamas, May 2006
- ✚ **Saghari Fard MR***, Poynton SL “Flagellar attachment, colony formation and encystment in *Spironucleus salmonis* (Diplomonadidae); new targets for treatment?” at 5th International Symposium on Aquatic Animal Health (ISAAH), San Francisco, California, September 2006
- ✚ Poynton SL*, Cheng J, **Saghari Fard MR**, Knopf K “Testing susceptibility to diplomonad infection; development of a new plasma incubation test” at 5th International Symposium on Aquatic Animal Health (ISAAH), San Francisco, California, September 2006

* **Presenter**

¹ **The winner of a student travel award**

² **The winner of second prize for graduate student oral presentation**

DISSERTATION OVERVIEW

The focus of the present study is on a diplomonad flagellate, *Spirotrunculus salmositica*, which infected rainbow trout in a farm near Berlin. First, I characterised the species of flagellate by using scanning and transmission electron microscopy; for molecular characterisation, the samples were sent to my colleagues Dr. Sterud and Dr. Jørgensen in National Veterinary Institute in Oslo, Norway. Second, I studied the intestinal pH of rainbow trout as a possible factor determining the microhabitat of the flagellate. Thirdly, I cultured the organisms, and recorded the clusters of trophozoites facilitated by adhesion of the posterior flagella prior to encystment. Fourthly, I conducted a quantitative study on the intestinal histopathology associated with the infection by comparing parameters in moderately infected with uninfected fish. Finally, I performed the modified *in vitro* plasma incubations test which is used for detecting the host susceptibility to pathogens.

A short explanation about contents of each chapter is given as follows;

Chapter 1, overview of diplomonads: provides an overview of free-living and parasitic diplomonads, including taxonomy, phylogeny, morphology and ultrastructure, habitat, life cycle and reproduction, pathology, immunity, and management and treatment of infection.

Chapter 2, piscine diplomonad, *Spirotrunculus*: focuses on diplomonads in fish. The chapter considers the effects of diplomonad infection in aquaculture, explains which genera and species of piscine diplomonads are well characterised, describes their ultrastructural morphology, discusses the microhabitat preferences of piscine diplomonads, their life cycle – trophozoites and cysts, encystment – the role of flagella, histopathology, the innate and acquired immunity.

Chapter 3, specific aims: presents the specific aims of the present study, which were pursued during my investigations.

Chapter 4, materials and methods: comprise the materials and methods, which I used during my study.

Chapter 5, results and discussion: comprise the results and discussions of the following aspects of my study; characterisation of *S. salmonis*, intestinal pH and microhabitat preference of *S. salmonis*, multi-functionality of flagella in *S. salmonis*, pathology associated with *S. salmonis*, and *in vitro* plasma incubation test for determining host susceptibility to *S. salmonis*.

Chapter 6, synthesis and critiques: comprise synthesis and critiques of all aspects of the present research. In this chapter I explain that how my study adds value and new knowledge to the topic of piscine diplomonads, and highlights key results that impact other areas of protozoology. I also recommend further essential studies for better understanding and managing of this pathogen in fish.

Chapter 7, literature: lists all the literature cited in this dissertation.

ABSTRACT

Parasitic diseases pose a significant threat to aquaculture, and parasitic diplomonad flagellates *Spironucleus salmonis* in rainbow trout, *Oncorhynchus mykiss* are associated with morbidity and mortality. Their management needs characterisation of the parasite, and understanding of host-parasite interactions, and the diplomonad in Germany has not been thoroughly studied. I fixed diplomonads in glutaraldehyde, and characterised the species by ultrastructure morphology (SEM and TEM). For microhabitat preference, I recorded occurrence and density, and pH, in the pyloric, anterior, middle, and posterior intestinal regions. For encystment, I cultured diplomonads in MEM with bovine serum or newborn calf serum for yielding trophozoites and cysts respectively; then I described encystment using light and SEM. For pathology, pyloric regions of intestine, and liver, of uninfected and moderately infected fish, were sectioned, stained with H&E, and PAS/AB. I recorded 6 pathology parameters, and developed a quantitative protocol for determining hyperplasia/hypertrophy of goblet cells. For immunity, I refined the *in vitro* plasma incubation test, for detecting susceptibility of rainbow trout, carp, and sturgeon to *S. salmonis*. Ultrastructural characterisation revealed *Spironucleus salmonis*, allowed its complete description including newly showing the caudal projection, discharging vacuoles, and deformable nuclear lobes; diagnostic keys were improved. The ssu rRNA gene sequence (investigated by Norwegian colleagues) distinguished *S. salmonis* from other piscine *Spironucleus* spp.. Occurrence and density of *S. salmonis* were significant higher in the pyloric region than elsewhere. The pH profile in uninfected and infected fish was similar: pyloric and posterior regions mean 7.3 and 7.1, anterior and middle regions mean 7.7 in both. A causal relationship between microhabitat preference and pH was unlikely; the optimal pH for *S. salmonis* was 7.1 – 7.5. In the MEM newborn calf serum media, clustering and encystment began by trophozoites attaching at tip of adhesive posterior flagella to each other or debris. By additional attachment of posterior flagella from other trophozoites, there were clusters of 50 trophozoites. Pyriform trophozoites became sub-spherical, anterior flagella inactive, surface blebs produced a refractile cyst wall. This is the first report of multifunctionality of flagella in diplomonads. Cysts clusters *in vivo* may be buoyant, and exceed

minimum infective dose for new infection. There was significant hypertrophy and hyperactivity of goblet cells in infected fish, but no hyperplasia. This hyper-production of mucus may decrease nutrient absorption, underlying impaired growth in *S. salmonis* infected fish. Vacuolisation of enterocytes was significantly more common in infected fish, but hepatocellular necrosis was not seen. The plasma incubation test was successfully modified for enteric diplomonads, and preliminary results show that it predicts susceptibility to *S. salmonis*. The hierarchy of resistance of *S. salmonis* was sturgeon > carp > rainbow trout; this parallels epizootiological data. My research yielded unexpected results of practical application within and beyond fish parasitology: new targets for new species-specific diagnostic tests (loss of ribosomes, cytoskeleton), improved *in vitro* conditions (pH 7.1-7.5), new therapeutic approaches (to reduce mucus, anti-adhesion therapy), and the potential to use cultures of *S. salmonis* for investigating multi-functionality of flagella and flagellar signalling.

CHAPTER 1

OVERVIEW OF DIPLOMONADS

CHAPTER 1

Overview of diplomonads

1.1 Taxonomy

According to the traditional five kingdom system of Whittaker (1969), and six kingdom system of Cavalier-Smith (1998), the diplomonads have been classified in the kingdom Protozoa, and grouped in the subphylum “Eopharyngia” within the phylum “Metamonada” of the subkingdom “Archezoa” Cavalier-Smith (1998).

Six kingdom system of life, Cavalier-Smith (1998)

1. Kingdom Bacteria, the sole primary kingdom of life
2. Kingdom Fungi, and its 4 phyla
3. Kingdom Plantae, and its 5 phyla
4. Kingdom Chromista, and its 5 phyla
5. Kingdom Animalia, and its 23 phyla
6. **Kingdom Protozoa, and its 13 phyla**

Subkingdom 1. Archezoa

Phylum 1. Metamonada

Subphylum 1. Eopharyngia (incl. diplomonads)

Phylum 2. Trichozoa

Subkingdom 2. Neozoa

Infrakingdom 1. Sarcomastigota

Phylum 1. Neomonada

Phylum 2. Cercozoa

Phylum 3. Foraminifera

Phylum 4. Amoebozoa

Infrakingdom 2. Discicristata

Phylum 1. Percolozoa

Phylum 2. Euglenozoa

Infrakingdom 3. Alveolata

Phylum 1. Dinozoa

Phylum 2. Sporozoa

Phylum 3. Ciliophora

Infrakingdom 4. Actinopoda

Phylum 1. Heliozoa

Phylum 2. Radiozoa

In recent years there have been great improvements in the highest ranks in taxonomic life system of eukaryotes, thanks to ultrastructural studies and molecular phylogenetics. The traditional six kingdom system has now been changed to a new scheme of six eukaryote groups based on phylogenetic taxonomy (Berger 2002). Eukaryotes are divided now into 6 major groups; (1) the Opisthokonta, grouping the animals, fungi, choanoflagellates, and Ichthyosporea; (2) the Amoebozoa, grouping most traditional amoebae, and slime moulds; (3) the Plantae, grouping the Glaucophyta, red algae, green algae, and plants; (4) the Chromalveolata, grouping the Alveolata (ciliates, dinoflagellates, Apicomplexa), the Stramenopiles (brown algae, diatoms, many zoosporic fungi), with the Haptophyta and Cryptophyceae; (5) the Rhizaria, grouping the Foraminifera, most of the traditional Radiolaria, and the Cercozoa; (6) the Excavata, grouping oxymonads, parabasalids, diplomonads, jakobids, the Euglenozoa and Heterolobosea and several other genera of heterotrophic flagellates (Adl et al. 2005, Simpson and Roger 2002, 2004) (Fig. 1.1).

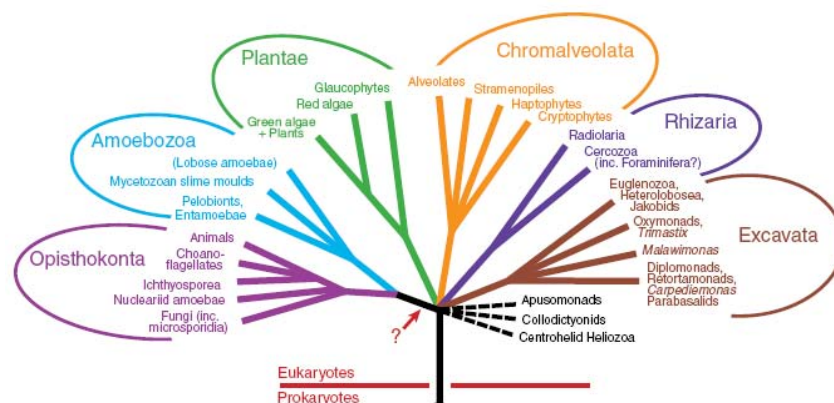


Fig. 1.1. A diagrammatic tree assigning eukaryotes into 6 major groups (from Simpson and Roger 2004). Diplomonads are in Excavata.

The order Diplomonadida [Excavata: Fornicata: Eopharyngia] comprising some suborders, families, subfamilies, and genera, which have recently been modified according to ultrastructural features, and were presented by Brugerolle and Lee (2002) in the book “The Illustrated Guide to the Protozoa” as shown below (Fig. 1.2).

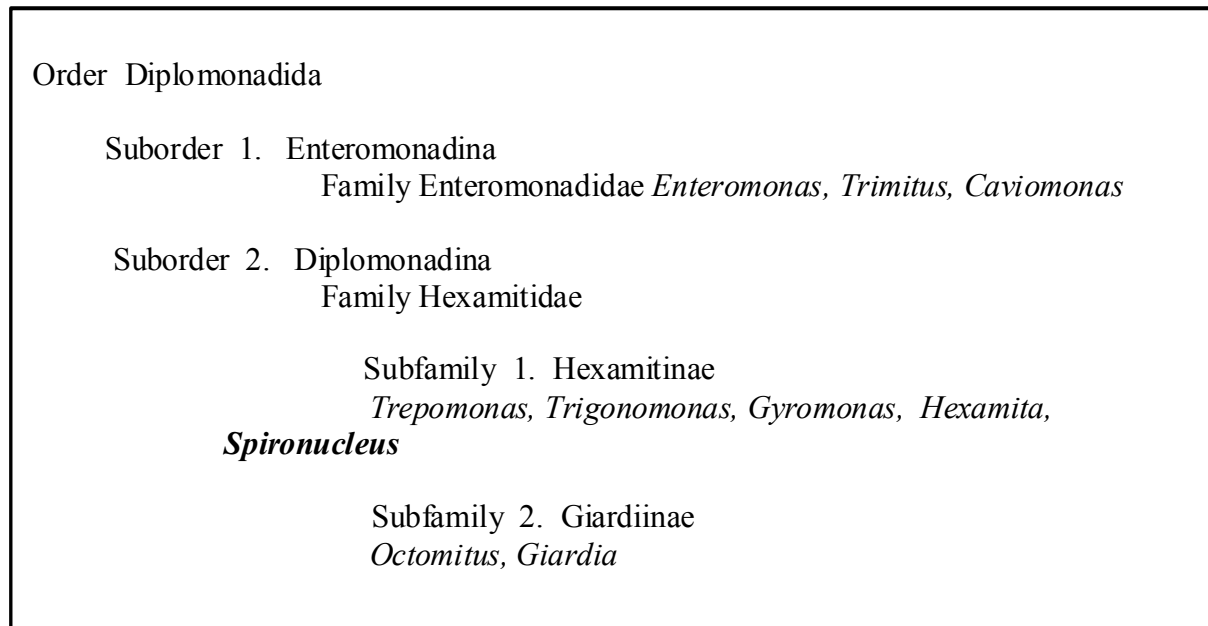


Fig. 1.2. Lower taxonomic ranking of the order Diplomonadida according to Brugerolle and Lee (2002).

1.2 Phylogeny

In molecular phylogenetics, the small subunit ribosomal RNA gene is now commonly use for understanding the relationship between organisms and their positions in the tree of life (Berger 2002). The most recent phylogenetic tree shows the position of diplomonads among major groups of eukaryotes, and particularly among Excavata (Embley and Martin 2006) (Fig. 1.3).

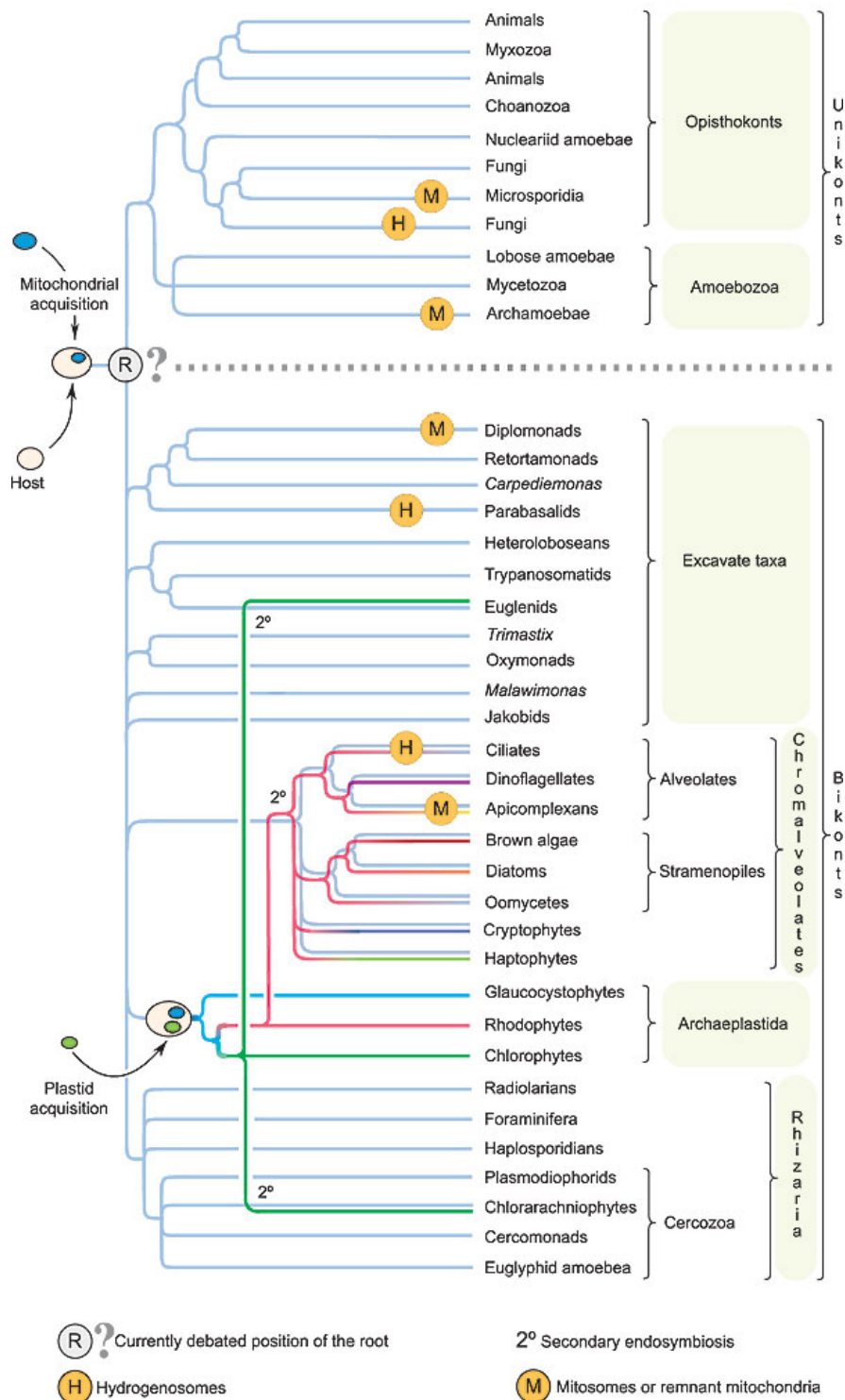


Fig. 1.3. The schematic tree of the relationship among major groups of eukaryotes (from Embley and Martin 2006). This is a composite tree. Note the position of the diplomonads in the Excavata.

The small-subunit ribosomal RNA gene sequences show Diplomonadida as a well supported clade (a clade is a taxonomic group comprising a single common ancestor and all the descendants of that ancestor) (Jørgensen and Sterud 2006). Diplomonads are a sister group to the retortamonads (Embley and Martin 2006), and both diplomonads and retortamonads are rooted by genus *Carpodomonas* (Embley and Martin 2006, Jørgensen and Sterud 2006, Simpson et al. 2002).

Within the order Diplomonadida, it was believed that the suborder of enteromonads were more primitive than the suborder of diplomonads, based on morphological characters and the degree of parasitism of their genera (Brugerolle 1975). The recent phylogenetic studies based on small subunit ribosomal RNA gene sequences, recovered enteromonads as a derived lineage (Kolisko et al. 2005), and Giardiinae (diplomonads) on a basal branch of the diplomonad root (Keeling and Brugerolle 2006).

The small subunit ribosomal RNA gene sequences are available for 15 diplomonad species. The species for which sequence data is available in public databases from GenBank, the European Ribosomal RNA Database (<http://www.psb.ugent.be/RNA/>), and other recent phylogenetics sources are listed below:

Enteromonads

Trimitus sp.

Diplomonads - Giardiinae

Giardia ardeae, *G. lamblia*, *G. microti*, *G. muris*

Octomitus intestinalis

Diplomonads - Hexamitinae

Trepomonas agilis,

Hexamita inflata, *H. nelsoni*

Spironucleus barkhanus, *S. meleagridis*, *S. muris*, *S. salmonicida*, *S. torosa*, *S. vortens*

In addition to the small subunit ribosomal RNA gene, α -tubulin, β -tubulin, *ef-1 α* (elongation factor-1 α), actin, *hsp70* and *hsp90* (heat shock proteins 70 and 90) are used in molecular phylogenetics (Harper et al. 2005). In diplomonads, Keeling and Doolittle (1996, 1997) used *ef-1 α* , α -tubulin, *eIF-2 γ* (gamma subunit of translation initiation factor-2) to define the distribution of genetic codes within the diplomonads. The *ef-1 α* (elongation factor-1 α), *gdh* (glutamate dehydrogenase), and *tpi* (triose phosphate isomerase) have been applied to obtain adequate species-level taxonomy of the diplomonad genus *Giardia*, which has been shown to contain at least six genetically distinct *Giardia* species (Adams et al. 2004, Monis et al. 1999, Thompson and Monis 2004).

It is worth noting that recent papers have shown phylogenetic trees with unresolved origins of many lineages, and many alternative possibilities still exist regarding the root of the tree of the Eukaryota (Baldauf 2003, Roger and Silberman 2002, Simpson and Roger 2002). For many years, diplomonads were thought to belong to the earliest-diverging lineage within the eukaryotes, due to lack of mitochondria in their cytoplasm, and they were believed to be one of the most primitive eukaryotes and perhaps represent an intermediate for the prokaryote-to-eukaryote transition (Cavalier-Smith 1993, Leipe et al. 1993, Van Keulen et al. 1992).

However, the advances in molecular phylogenetics and cell biology during the last decade strongly suggest this view is incorrect (Baldauf 2003, Embley and Martin 2006, Simpson and Roger 2004). In addition, the finding of some sort of mitochondria in diplomonads was a turning point for views of early eukaryotic and mitochondrial evolution. Tovar et al. (2003) recently showed that *Giardia* (a human pathogenic diplomonad) has highly reduced mitochondria which are called mitosomes. Unlike conventional mitochondria, *Giardia's* mitosomes do not generate ATP for energy, instead they assemble iron-sulphur (Fe-S) clusters for making ATP. Thus *Giardia's* place as an intermediate stage in standard scheme of eukaryotic evolutionary history is no longer tenable (Embley et al. 2003, Tovar et al. 2003).

1.3 Morphology and ultrastructure

Since members of the Diplomonadida are very small, less than 30 micron in length, electron microscopy is essential in order to visualize details of their structure. Most of the early descriptive studies made using light microscopy are now regarded as limited and unreliable (Poynton and Sterud 2002, Brugerolle and Lee 2002), (these are discussed further in section 2.2 "Identification of *Spironucleus* species in fish - chronology").

On the basis of ultrastructural data, Brugerolle and Lee (2002) created two suborders (Enteromonadina and Diplomonadina) within the order Diplomonadida. The structure of the enteromonad cytoskeleton is very similar to that of diplomonads, apart from the fact that it is not duplicated. Thus an enteromonad (monozoic form) can be described as one half of the cell of a diplomonad (diplozoic form). The cell basically has a flagellar (mastigont) system with 1 or 2 karyomastigonants each of which consists of 1 nucleus, 4 basal bodies (kinetosomes), and 1-4 flagella, of which one flagellum is always directed posteriorly (recurrent flagellum), and may be associated with a cytostomal canal; there are 3 sets of microtubules, distinguished by their passage above the nucleus (supra-nuclear microtubules), under the nucleus (infra-nuclear microtubules), and along the recurrent flagellum (cytostomal microtubules/direct microtubules/funis) (Fig. 1.4).

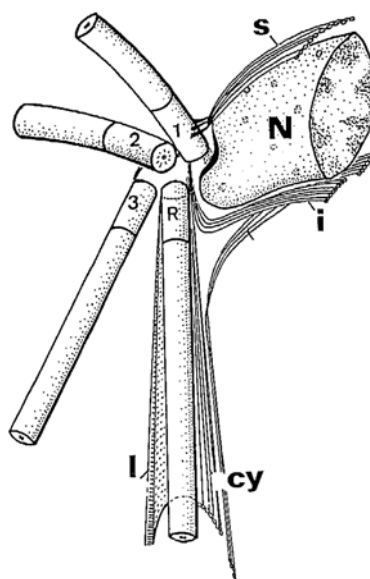


Fig. 1.4. A karyomastigont; nucleus (N), basal bodies (1,2,3,R), supra-nuclear microtubules (s), infra-nuclear microtubules (i), cytosomal microtubules (also called direct microtubules, funis) (cy), striated lamina (l), (from Brugerolle and Lee 2002).

Within the Order Diplomonadida, 3 genera in the suborder Enteromonadina; *Enteromonas*, *Trimitus* and *Caviomonas*, and 7 genera in the suborder Diplomonadina; *Trepomonas*, *Trigonomonas*, *Gyromonas*, *Hexamita*, *Spironucleus*, *Octomitus*, and *Giardia*, are ultrastructurally well studied, and a key has been produced by Brugerolle and Lee (2002).

Key to Suborders, Families, Subfamilies and Genera of the Diplomonadida (Brugerolle and Lee 2002);

1	Monozoic organism (with one karyomastigont).....	Suborder Enteromonadina	
Family Enteromonadidae		2
1'	Diplozoic organism (with two karyomastigonts).....	Suborder Diplomonadina	
Family Hexamitidae		4
2	With four flagella.....	<i>Enteromonas</i>	
2'	With three or less flagella.....		3
3	With three flagella (one recurrent and two anterior flagella).....	<i>Trimitus</i>	
3'	With one flagellum.....	<i>Caviomonas</i>	
4	With two posterior oral grooves.....		5
4'	With two posterior cytosomal canals.....		6
4''	No posterior oral grooves or cytosomal canal.....		7
5	Oral grooves containing three recurrent flagella.....	<i>Trepomonas</i>	
5'	Oral grooves with only two flagella.....	<i>Trigonomonas</i>	
5''	Oral grooves with only one flagellum.....	<i>Gyromonas</i>	

- 6 Flagella inserted on the external side of the nuclei, endoplasmic reticulum associated with the cytostomal canal.....*Hexamita*
- 6' Flagella inserted at the apex, s-shaped nuclei, no endoplasmic reticulum associated with the cytostomal canal.....*Spironucleus*
- 7 Central axis formed by two recurrent axonemes, microtubular fibers and endoplasmic reticulum.....*Octomitus*
- 7' With a ventral disk.....*Giardia*

In the same year that Brugerolle and Lee (2002) published their key on free-living and parasitic diplomonads, Poynton and Sterud (2002) offered a more detailed ultrastructural key for distinguishing the three genera of diplomonads, *Hexamita*, *Octomitus*, and *Spironucleus*, previously reported from fish. Poynton and Sterud (2002) focused more on the shape of nuclei, and the position of the recurrent flagella relative to nuclei than did the schemes of Brugerolle and Lee (2002).

Key to genera of diplomonads reported from fish (Poynton and Sterud 2002).

- 1 With two posterior flagellar pockets (cytostomal canals).....2
- 1' Without posterior flagellar pockets (cytostomal canals).....3
- 2 Kinetosomes on external surface of spherical nuclei, recurrent flagella pass posteriorly laterally over the nuclei.....*Hexamita*
- 2' Kinetosomes just below apex of s-shaped nuclei, recurrent flagella pass posteriorly medial to nuclei.....*Spironucleus*
- 3 Central axis formed by recurrent axonemes, microtubular bands and endoplasmic reticulum; nuclei reniform.....*Octomitus*

1.4 Habitat

Simpson and Roger (2004) described Excavata as follows: “Excavata are unicellular eukaryotes, most of which are heterotrophic flagellates. They include several groups that cause significant diseases, such as trypanosomatids, **diplomonads**, and parabasalids.....but each parasitic group has free-living relatives....”

The three known genera in the family Enteromonadidae, *Enteromonas*, *Trimitus* and *Caviomonas*, inhabit the intestine of both vertebrates and invertebrates as harmless commensals. *Enteromonas hominis* inhabits the caecum of man, monkeys, rodents and rabbits (Goldberg 1990, Kulda and Nohýnková 1978, Spriegel et al. 1989). *Trimitus* lives in the intestine of the insect, fish, frog, snake, and tortoise (Brugerolle and Lee 2002). *Caviomonas mobilis* has been found in the caecum of the guinea-pig (Brugerolle and Lee 2002).

Among the 7 genera in the family Hexamitidae, two genera are free living (*Trepomonas*, and *Trigonomonas* - except *Trepomonas agilis* which is parasitic in the intestine of amphibian, fish, and tortoise), one genus (*Hexamita*) contains free living and parasitic species, and three genera are parasitic (*Giardia*, *Octomitus*, *Spironucleus*) (Brugerolle and Lee 2002, Kulda and Nohýnková 1978, Siddall et al. 1992). *Gyromonas* are reported only by light microscopy, and appear to be little studied.

The free living diplomonads, such as *Hexamita inflata*, are found in waters rich in organic matter and deficient in oxygen, such as sediment, stagnant reservoirs, marshes, water treatment plants, and also in brackish or salt water. The flagellates swim actively and feed on bacteria, and dead cells of other protozoans, plants, and animals which are engulfed by the cytostomal canal (Biangini et al. 1997, 1998, Brugerolle and Lee 2002, Fenchel et al. 1995).

The parasitic species of *Hexamita* occur in the digestive tract of various vertebrates such as *H. cryptocerci* in insects, *H. nelsoni* in oysters, *H. teres* in rodents, and *H. pitheci* in monkeys (Brugerolle and Lee 2002). The report of *H. salmonis* from intestine of fish is no longer considered correct (Poynton et al. 2004).

The parasitic diplomonads are mostly common inhabitants of the alimentary tract of hosts, but they have been seen in other organs (Brugerolle and Lee 2002, Kulda and Nohýnková 1978, Woo 2006). Most of them are commensals feeding on bacteria and on food digested by the host. However, some of them are pathogenic.

The species of *Giardia* are reported in the alimentary tract of various vertebrates such as *G. agilis* in amphibians, *G. muris* in rodents, and *G. lamblia* in mammals (humans, dog, cow, sheep, goat, rabbit, chinchilla) (Brugerolle and Lee 2002, Faubert 2000).

The species of *Octomitus* are parasitic in the intestine of vertebrates such as *O. neglecta* in amphibians, *O. intestinalis* in rodents (Brugerolle and Lee 2002), and reptiles (Tomova and Golemansky 2001).

The species of *Spironucleus* live in the intestine of various vertebrates such as *S. elegans* in amphibians, *S. muris* in mice, and *S. meleagridis* in game birds and turkeys (Brugerolle and Lee 2002, Cooper et al. 2004). Among piscine *Spironucleus* species, some have only been reported from the intestine such as *S. torosa* (Poynton and Morrison 1990), while others have been reported from extra-intestinal locations such as muscle and blood, for example *S. salmonicida* (Jørgensen and Sterud 2004, 2006). *Spironucleus* (previously known as *Hexamita*, *Octomitus*) has also reported in reptiles (Frank 1984).

Diplomonads have shown different microhabitat preferences in the alimentary tract of the host, for example *S. torosa* prefer the rectum of fish (Poynton and Morrison 1990, Sterud 1998a,b) whereas *S. salmonis* is commonly found in pyloric region of the intestine of fish (Poynton et al. 2004). Although diplomonads are usually extra-cellular, some may attach to the host cells like *G. lamblia* which their ventral side has been modified into a sucking disc and used for attachment to the small intestinal mucosa of human (Faubert 2000). The intra-cellular infection of a diplomonad (*S. salmonicida*, previously known as *S. barkhanus*) has been recently reported in capillaries and sinusoids of the liver, spleen, and head kidney of farmed Arctic char (Sterud et al. 2003). In the systemic form of infections, diplomonads like *S. salmonicida* in fish distribute in other host organs including blood (Jørgensen and Sterud

2004) (the possible factors effecting microhabitat preferences are discussed further in section 2.3 “Habitat of *Spironucleus*”).

1.5 Life cycle - reproduction

There are two stages in the diplomonad life cycle, trophozoites and cysts. The trophozoite is the motile stage that actively feeds and multiplies, and for parasitic species, this is the stage that is most readily observed, usually in the intestinal lumen (for a description see section 1.3 “Morphology and ultrastructure”). The cyst is the resistant stage of life cycle in which the cell can survive outside of the host (Woo 2006). These two stages occur in all genera of diplomonads, and in both free living and parasitic forms (Brugerolle and Lee 2002, Kulda and Nohýnková 1978).

Trophozoites and cysts pass out of the host with the feces, and are ingested orally by the host. Although the faecal-oral transfer of diplomonads cysts is the major route of transmission in fish, infection via the trophozoites from skin lesions in Atlantic salmon (Poppe et al. 1992), and the rectal route of transmission through cyst and trophozoites were also suggested (Kent et al. 1992, Kulda and Lom 1964, Moore 1922a,b, Poynton and Morrison 1990).

Trophozoites reproduce asexually by longitudinal binary fission. In the cysts, binary division probably occurs in all diplomonad genera (Kulda and Nohýnková 1978, Siddal et al. 1992). Most studies on diplomonads cysts were focused on *Giardia lamblia* from humans, because of their importance in medicine (Adam 2001), however some studies has also been done on *S. muris* from mice, and *S. meleagridis* from birds, because of their importance as pathogens in laboratory and commercial animals respectively.

In *G. lamblia* from humans, cysts were approximately 5 x 7 to 10 µm in diameter, covered by a wall that was 0.3-0.5 µm thick, and contained two flagellates with 4 nuclei (2 nuclei for each flagellate) (Adam 2001). The cysts of *S. muris* from mice were 7.5-13 x 4.5-6 µm in diameter,

but no information about thickness of cyst wall was given (Brugerolle et al. 1980). However, the cyst wall in *S. meleagridis* from birds was measured, and was approximately 0.3 µm thick (Wood and Smith 2005). The cysts in *S. meleagridis* contained 2 flagellates with 4 nuclei; however a single trophozoite with 2 nuclei within the *S. muris* cyst, and the cysts with 2 or 3 nuclei in *S. meleagridis* were also observed (Brugerolle et al. 1980, Wood and Smith 2005). The details of the cyst will be discussed further in section 2.5 “Encystment- the role of flagella”.

1.6 Pathology

Of all 10 well-known genera of diplomonads, four are recognized as pathogens, namely *Hexamita* and *Spirotrunculus* from subfamily Hexamitinae, and *Giardia* and *Octomitus* from subfamily Giardiinae (Brugerolle and Lee 2002, Woo 2006). Among these four genera, *Giardia* and *Spirotrunculus* have received the most attention, because of their importance in human and veterinary medicine respectively.

A striking feature of giardiasis is the uneven presentation of clinical symptoms, ranging from asymptomatic to chronic disease, with associated diarrhoea, malabsorption and weight loss (Adam 2001). Among *Giardia* species; *G. lamblia*, and *G. muris* are well known pathogens affecting human, and mice. The histopathological changes occurring at the intestinal mucosa of humans, where *G. lamblia* trophozoites attach, range from minimal to severe enough to cause enteropathy with enterocyte damage, villus atrophy, and crypt hyperplasia (Ferguson et al. 1990, Faubert 2000). *Giardia muris* infection in mice is associated with weight loss, stunted growth, rough coat, and enlarged abdomen. Pathologic changes include villous blunting, increase number of intra-epithelial lymphocytes, goblet and mast cells, and alterations in intestinal disaccharides (Venkatesan et al. 1997).

Spirotrunculosis (hexamitiasis or hexamitosis) is well studied in *S. meleagridis*, and *S. muris*, which affect birds, and mice respectively. *S. meleagridis* (syn. *Hexamita meleagridis*) affects turkeys and game birds including chukar partridges, quail and peafowl (Barnes et al. 2003). The intestinal infection is associated with watery diarrhoea, loose fecal droppings,

emaciation, depression, dermatitis of the face and legs, and rapid weight loss (Cooper et al. 2004, Lloyd et al. 2005). Although villous atrophy, and inflammation are absent (Hussain 2001, Lloyd et al. 2005), moderate enteritis with fusion of villi, with inflammatory cells, has been reported from chukar partridges (Cooper et al. 2004). *S. muris* in mice mostly is asymptomatic; however it has been associated with diarrhoea, dehydration, weight loss, and abdominal distension (National Research Council 1991, Whitehouse et al. 1993). The crypts are hyperplastic and might be distended with trophozoites, microvilli and villi might be shortened, and enterocyte turnover is increased with minimal inflammation (National Research Council 1991, Whitehouse et al. 1993).

Among well known piscine species of *Spironucleus* in fish, *S. barkhanus*, *S. salmonis*, and *S. torosa* are common in the digestive tract and gall bladder, and are less pathogenic parasites in contrast with *S. salmonicida*, and *S. vortens*, which cause systemic infection in fish mostly in aquaculture. However there is a recent report of systemic infection of *Spironucleus* (presumably *S. salmonicida*) in wild fish too (Meseck et al. 2007). The other reports of diplomonad infection in fish reported as *Octomitus*, and *Hexamita*, are no longer considered reliable and valid. The only ultrastructurally known piscine diplomonad is *Spironucleus* (Poynton and Sterud 2002, Poynton et al. 2004) (more details in section 2.2 “Identification of *Spironucleus* species in fish – chronology”).

S. salmonis found in intestine of rainbow trout cause weakness, anorexia, and emaciate in fish (Naich and Bilgees 1992). Internally, enteritis, intestinal haemorrhage, yellow mucus in the intestine, and necrosis of hepatocytes may be observed (Woo 2006). *S. salmonicida* (previously *S. barkhanus*) is associated with systemic infection in farmed Arctic char, Atlantic salmon, and Chinook salmon. It is found not only in intestine and gall bladder, but in liver, kidney, spleen, and muscle. The pathology associated with infection was muscle lesions, necrosis in liver kidney and spleen, and haemorrhages in the intestine (Kent et al. 1992, Meseck et al. 2007, Poope et al. 1992). The pathology associated with *Spironucleus* infection in fish is discussed in more detail in section 2.6 “Pathology associated with *Spironucleus*”.

1.7 Immunity

The immunity of a host to a pathogen can be distinguished into recognition and response. Recognition involves distinguishing foreign pathogens from another, or distinguishing the foreign molecules from the host cells. After recognition, two forms of immunity, innate and acquired, work in concert to respond to pathogens (Goldsby et al. 2003, Woo 1996). While invertebrates rely only on the innate immunity system, higher vertebrates have both innate and acquired immune systems (Benjamini et al. 2000, Van Muiswinkel 1995).

Innate immunity is the natural resistance to an infection or disease, and can be considered as physiological barriers (e.g. low pH in the stomach which kills ingested micro-organisms), anatomical barriers (e.g. skin surface), humoral components (e.g. enzymes, proteins and complements), and cellular components (e.g. macrophages, and granulocytes) (Evelynn 1996, Goldsby et al. 2003, Van Muiswinkel 1995, Yano 1996).

Acquired immunity is more specialized than innate immunity, and the hosts that survive infection by using an effective acquired immune system are generally immune to further subsequent attack of the same pathogen (Benjamini et al. 2000). However, acquired immunity is usually slower to develop in poikilothermic hosts (e.g. fish) than in homeothermic hosts. This might be due to the lower body temperatures and slower metabolic rates of the former (Woo 1996). The acquired immune system comprises cell-mediated response (T-cells, B-cells, and antigen-presenting-cells), and humoral response (antibodies or immunoglobulins which are produced by B-cells).

Immunity tests can detect and analyze the quality and quantity of both innate and acquired immunity. The test for acquired immunity is based on the antigen-antibody interaction, which include for example the enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA), lymphocyte function assay, and complement assay (Benjamini et al. 2000).

The innate immunity tests like the *in vitro* plasma incubation test determine what happens when resistant and susceptible hosts are exposed to the pathogen. Host susceptibility and

innate immunity can be considered at two distinct levels - between species and within species. Between species innate immunity means the resistance that occurs at the host species (or a higher taxonomic group) level. Within species innate immunity means the resistance that occurs in individuals within a susceptible host species (Woo 1996).

The immunity of hosts to diplomonad infection is rather unevenly known, and although there have been intensive studies on the genus *Giardia* (a significant pathogen of humans and rodents), little is known about immunity to other diplomonad species. For example, in *Giardia lamblia*, the innate immunity plays a role in the control and/or severity of the infection in the intestine of humans. *G. duodenalis* trophozoites are killed by products of lipolysis present in human intestinal fluid (Faubert 2000). Aley et al. (1994) have also reported that human antimicrobial peptides (such as defensins) in the human intestine have anti-trophozoite activities. Both humoral and cell-mediated responses play a role in acquired immunity to *Giardia lamblia*. Human serum that containing anti-*G. lamblia* antibodies (humoral response - classical pathway of complement) lysed the trophozoites, and lymphocytes were found in large numbers in the epithelial layer (cell-mediated response) (Faubert 2000). The immunity in piscine diplomonads will be discussed in details in section 2.7 "Innate immunity and host susceptibility".

1.8 Management and treatment of infection

For piscine *Spironucleus*, metronidazole can be used to treat the infection, and is available commercially in fish food flakes. The metronidazole kills diplomonads by destroying their DNA. A dose of 5 g metronidazole per kg feed for 2 days can be used in the fish food (Stoskopf 1993, Tojo and Santamarina 1998). A bath of metronidazole by immersing the fish for 6 to 12 hours, with 250 mg of metronidazole per 10 gallons of water can also be used instead of oral treatment (Tojo and Santamarina 1998). For *Giardia*, metronidazole has also shown to be efficacious against infection in other vertebrates including human, mice, and birds (Samuelson 1999). No treatment has been yet suggested against diplomonad cysts.

Prevention of piscine diplomonad infection can be achieved by good husbandry and nutrition, removing infected fish and disinfecting the pond bottom with quicklime (Schäperclaus 1991, Stoskopf 1993, Woo 2006). However no specific vaccination has been suggested for diplomonad infection yet.

Although metronidazole is a well known oral treatment against diplomonad infections that can be given in the food, it is not allowed to be used in food fish in Europe (Buchmann and Bresciani 2001), because of serious side effects and carcinogenicity. The resistance to metronidazole has also been reported in some fish farms (Tojo and Santamarina 1998). All of these challenges show that alternative treatment approaches for diplomonad infection in aquaculture are critical.

CHAPTER 2

PISCINE DIPLOMONAD

SPIRONUCLEUS

CHAPTER 2

Piscine diplomonad: *Spironucleus*

2.1 Aquaculture and *Spironucleus*

Aquaculture is a crucial element of food supply worldwide. Decline in wild catch, coupled with increasing human population, means that adequate protein supply can only be met by production from aquaculture. It is estimated that production from the aquaculture industry needs to be doubled in the next 50 years, to meet this demand (FAO 2006).

According to FAO statistics, China is the biggest producer in aquaculture production in terms of quantity (Iran: in terms of growth), and carp is the top species in terms of quantity of fish production worldwide (FAO 2006). Other commonly cultured fish species include: tilapia, catfish, sea bass, bream, salmon and trout. Salmonid aquaculture is a major economic contributor to the world production of farmed fin-fish, representing over \$1 billion US annually (FAO 2006). Salmon farming is a significant element of food production in Chile, Norway, Scotland, Canada, and is the source for most salmon consumed in America and Europe (FAO 2006). Trout is also a well-known production fish worldwide; taking about 7.4% of estimated global use of fishmeal within aqua-feeds, which is very large to compare with the 14.9% for carp (FAO 2006). Rainbow trout *Oncorhynchus mykiss* is the largest sector in German fish farming, with production of 25, 000 t per year, which places the country among the top ten producers of rainbow trout worldwide (Brämick 2004, Hilge 2004).

However, a big challenge facing aquaculture, including that of rainbow trout, is diseases including those caused by parasites. Pathogenic intestinal protozoans, including diplomonads, are responsible for clinically important infections in fish worldwide. Diplomonads have been reported from wild, farmed and aquarium fishes in cold, temperate and warm waters. They have been found in various fish families mostly from cyprinids,

cichlids, salmonids, and gadids (Woo 2006). Diplomonad flagellates, of the genus *Spironucleus* (previously and mistakenly, named *Hexamita*), are commonly found in the digestive track of fish, and less commonly in systemic infections (Poynton and Sterud 2002). The pathogenic intestinal infections were reported in farmed fish with chronic clinical signs including poor appetite and weight loss (Ferguson 1979, Sterud et al. 1997, 1998, Uldal and Buchmann 1996). The pathogenic systemic infections occurred in farmed adult salmonids, with acute signs including muscle lesions, and ascites (Kent et al. 1992, Poppe et al. 1992, Sterud et al. 1998, 2003). The hole in the head disease was also reported in cichlids, such as angelfish and discus by Paull and Matthews (2001). In 2007 there has been the first report of pathogenic systemic infection in wild salmonids in North America (Meseck et al. 2007).

2.2 Identification of *Spironucleus* species in fish - chronology

Diplomonad flagellates in fish were first reported from the sunbleak *Leucaspis delineatus* (Cyprinidae) in Germany by Seligo (1887). Subsequently, there was a long period in which diplomonads from fish were studied by light microscopy, leading to many misidentifications. For diplomonads from trout, the first report appears to be that of *Urophagus intestinalis* from farmed rainbow trout in Germany by Moroff (1903). Some 16 years later, these organisms were described from farmed trout in Germany, and referred to as *Octomitus intestinalis truttae* by Schmidt (1919). Shortly thereafter, *Octomitus salmonis* was described from a trout hatchery in the east coast of North America (type description by Moore (1922a), and elaborated upon by Moore 1922b, and by Davis (1926)). Moore (1922a,b) noted that the American species of trout diplomonad caused serious lesions in the intestinal epithelium and disease (weakness, watery intestinal contents, and death of fingerlings), whereas the European species was reported to be a commensal. A later report in the same year, by Wenyon (1926), showed that both Moore and Davis had found *Hexamita salmonis*, not *O. salmonis*. Subsequently, *Hexamita salmonis* became the commonly used name for diplomonads from salmonids.

The correct identity of the diplomonads described by the European and North American authors, and their possible synonymy, has been considered by a number of authors including Buchmann and Bresciani (2001), Kulda and Lom (1964), Poynton and Sterud (2002), and Woo (2006). Much of the confusion has arisen when diplomonads in salmonids have been studied by light microscopy alone; the organisms are small, less than 15 µm long, and light microscopy is inadequate. Germany was not exempt from this poor characterisation of piscine diplomonads. Since the first report of diplomonads from rainbow trout in Germany by Schmidt (1919), numerous subsequent investigations have been done based on light microscopy, including a study of the ecology, host specificity and variability of *Hexamita salmonis* (Sanzin 1965). Several widely used fish pathology texts in Germany refer to the flagellates in trout as *Hexamita* (*Octomitus*) *salmonis* (Schäperclaus et al. 1990, Schlotfeldt 1991), and note that they are associated with cattharal enteritis in salmonids (Roberts and Schlotfeldt 1985).

Some taxonomic progress was made in 1979 by Ferguson, who published preliminary ultrastructural work (SEM and TEM) on a diplomonad from juvenile rainbow trout in Northern Ireland. He referred to it *H. salmonis*, retaining the genus name used for other diplomonads from salmonids.

The first comprehensive scanning and transmission electron microscopy study of a piscine diplomonad was conducted on flagellates from Canadian gadids by Poynton and Morrison (1990). The authors newly described *Spironucleus torosa* from cod *Gadus morhua* and haddock *Melanogrammus aeglefinus*, and in so doing, made the first confirmation – by ultrastructure – of the genus *Spironucleus* in fish. *Spironucleus torosa* was subsequently confirmed from gadids including saithe, *Pollachius virens*, in Norway by Sterud (1998a,b). In the meantime, the two additional *Spironucleus* species from fish were ultrastructurally characterized and reported, *S. barkhanus* from salmonids including grayling *Thymallus thymallus*, Atlantic salmon *Salmo salar*, and Artic char *Salvelinus alpinus* (Sterud et al. 1997, 1998), and *S. vortens* from the cichlids including angelfish *Pterophyllum scalare*, and discus *Symphysodon discus*, and from the cyprinid the ide *Leuciscus leuciscus* (Paull and Matthews 2001, Poynton et al. 1995, Sterud and Poynton 2002) (Table 2.1).

Table 2.1. The well characterised *Spironucleus* spp. from fish including their host, location in the host, and geographic locality.

Species	Host	Location	Geographic locality	Literature source
<i>S. barkhanus</i>	Arctic char	Intestine, gall bladder	W,D Norway	Sterud et al. 1998
	Grayling	Intestine, gall bladder	W,F Norway	Sterud et al. 1997
<i>S. salmonicida</i> ^a	Arctic char	Systemic (intracellular)	A,S Norway	Sterud et al. 2003
	Atlantic salmon	Systemic	A,S Norway	Sterud et al. 1997, 1998
	Chinook salmon	Systemic	A,S Canada	Kent et al. 1992
<i>S. salmonis</i> ^b	Brook trout	Intestine (pyloric)	A,F USA	Moore 1922a,b, Davis 1926
	Brown trout	Intestine (pyloric)	A,F USA	Moore 1922a,b, Davis 1926
	Lake trout	Intestine (pyloric)	A,F USA	Moore 1922a,b, Davis 1926
	Rainbow trout	Intestine (pyloric)	A,F USA	Ferguson 1979
	Rainbow trout	Intestine (pyloric)	A,F Ireland	Poynton et al. 2004
<i>S. torosa</i>	Burbot	Intestine (rectum)	W,F Norway	Sterud 1998a
	Cod	Intestine (rectum)	W,S Norway	Sterud 1998b
	Cod	Intestine (rectum)	W,S Canada	Poynton & Morrison 1990
	Haddock	Intestine (rectum)	W,S Canada	Poynton & Morrison 1990
	Saithe	Intestine (rectum)	W,S Norway	Sterud 1998b
<i>S. vortens</i>	Angelfish	Intestine (middle), lip tumor	A,F USA	Poynton et al. 1995
	Angelfish	Intestine, head lesions	A,F UK	Paull & Matthews 2001
	Discus	Systemic	A,F UK	Paull & Matthews 2001
	Ide	Intestine	W,D Norway	Sterud and Poynton 2002

A: aquaculture, D: anadromous, F: freshwater, S: seawater, W: wild

^a Diplomonads initially reported as a hexamitid infection by Kent et al. (1992), and as *S. barkhanus* by Sterud et al. (1997, 1998, 2003), and then renamed to *S. salmonicida* by Jørgensen and Sterud (2006).

^b Diplomonads initially reported as *Hexamita salmonis* by Moore (1922a,b), Davis (1926), and Ferguson (1979), and renamed to *S. salmonis* by Poynton et al. (2004).

In 2002, guidelines for species descriptions of diplomonads in fish were established by Poynton and Sterud (2002). In this comprehensive critique of the true identity of piscine diplomonads, Poynton and Sterud (2002) stated that the only reliable diagnostic methods for distinguishing diplomonads are transmission electron microscopy of determine genus, and elucidation of a suite of features seen by scanning and transmission electron microscopy, along with sequencing, to determine species. Poynton and Sterud (2002) suggest that, based on comprehensive electron microscopy observations, all diplomonads from fish belong to the genus *Spironucleus*.

Study of *Spironucleus* is not only of taxonomic relevance, but may also be important for understanding of host-parasite relationships. For example, it is known that *S. torosa* can enter into an intimate relationship with the rectal microvilli (Poynton and Morrison 1990), and intra-cellular infection of *S. salmonicida* in farmed Arctic charr are reported by Sterud et al. (2003), whereas *Hexamita* exist free in the lumen of host tissue (Poynton and Morrison 1990). The ability of *Spironucleus* to invade the mucosa, and localize in other tissues has also been pointed by Siddal et al. (1992).

In 2004, the hypothesis that piscine diplomonads belong to the genus *Spironucleus* was supported by a comprehensive TEM study that re-examined the diplomonad from the intestine of rainbow trout from Ireland originally studied by Ferguson (1979). The 2004 study (Poynton et al. 2004) resulted in *Hexamita salmonis* (Ferguson 1979) being synonymised with *Octomitus salmonis* Moore (1922a,b) and Davis (1926), and being renamed *Spironucleus salmonis* (Poynton et al. 2004). However, the 2004 study excluded both SEM studies and molecular characterisation of the parasite, thus key features remained unknown.

It is interesting to note that detailed electron microscopy studies of diplomonads from mice and birds (pheasants, and partridges) also have also moved diplomonad species from the genus *Hexamita* to the genus *Spironucleus* (Brugerolle et al. 1973, 1980, Kulda and Nohýnková 1978, Lloyd et al. 2005, Wood and Smith 2005).

Less detailed ultrastructural studies on diplomonads from fish, namely cyprinids in China, also suggest that the flagellates belong to the genus *Spironucleus*. Two new diplomonads, initially described as *Hexamita capsularis* and *Hexamita nobilis* (Li 1995, Xiao and Li 1994), most probably belong to *Spironucleus*. The published pictures show features characteristic of *Spironucleus*, namely anteriorly tapering and intertwined nuclei. To confirm their identity, these species should be examined based on guidelines provided by Poynton et al. (2004), and Poynton and Sterud (2002).

The most recently named *Spironucleus* species from fish, namely *S. salmonicida*, was identified genetically by sequence of the ssu rRNA from systemically infected sea-farmed Atlantic salmon, and Arctic charr (Jørgenson and Sterud 2004, 2006), hosts in which it was previously known as *S. barkhanus* (Sterud et al. 1997, 1998). The *S. salmonicida* isolate is associated with systemic and organ infections in cultivated salmon, whereas the morphologically identical but genetically distinct isolate (*S. barkhanus*) is present in benign intestinal and gall bladder infection in wild Arctic charr *Savelinus alpinus*, and wild grayling *Thymallus thymallus*. The rigorous studies by Jørgenson and Sterud (2004, 2006) elegantly demonstrate how molecular characterisation, in addition to ultrastructural characterisation, plays a key role in identifying diplomonad flagellates from fish, and linked genotypes with pathogenicity.

The small subunit ribosomal RNA sequencing on diplomonad flagellates from fish has recently been done on most ultrastructurally well-known piscine *Spironucleus* species; *S. barkhanus*, *S. salmonicida*, *S. salmonis*, *S. torosa* and *S. vortens* (Jørgensen and Sterud 2004, 2006), (more details in section 1.3 “Morphology and ultrastructure”).

Despite the advent of molecular studies of diplomonads from fish, this approach is in its infancy compared to the established molecular diagnosis for other parasites of economic importance in aquaculture such as microsporidia, myxosporea, and monogenea (Cunningham 2002).

2.3 Habitat of *Spironucleus*

Diplomonad infections in fish are mostly extra-cellular. However there are some reports of interaction of diplomonad trophozoites with host tissue; for example the attachment of *S. torosa* to the intestinal epithelium in cod without any development of systemic infection (Poynton and Morrison 1990). The observation of intra-cellular infections was in early report of *Octomitus salmonis* (renamed to *S. salmonis* by Poynton et al. 2004) in intestinal epithelial cells in trout by Moore (1922a,b) and Davis (1926). Since then, intra-cellular spironuclosis have not been reported until a recent report of *S. salmonicida* that can invade the mucosa and localise in other tissues in capillaries and sinusoids of the liver, spleen and head kidneys of farmed Arctic charr (i.e. flagellate in an erythrocyte, see Fig. 18 in Sterud et al. (2003)).

It was initially thought that *S. barkhanus* associated with both intestinal and systemic infection, however two species are now recognised, the intestinal and gall bladder isolate, and the systemic isolate, which has now been renamed as *S. salmonicida* (Jørgensen and Sterud 2004, 2006). Thus, the only *Spironucleus* species that can cause both enteric and systemic infection appears to be *S. vortens*; which cause an intestinal infection in angelfish, and a systemic infection in discus (Paull and Matthews 2001). However, this species has yet to be sequenced, so it is not known if different genotypes are associated with the different manifestations of the infection. Meseck et al. (2007) recently reported a case of systemic infection in wild Chinook salmon in North America, and they observed *Hexamita*-like sp., which presumably could be *S. salmonicida*.

S. barkhanus has been observed in the intestine and gall bladder of grayling, without any report of high concentration of *S. barkhanus* in specific region of the intestine (Sterud et al. 1997). Although the prevalence of infection in grayling was 100%, no sign of disease was observed in fish (Sterud et al. 1997). Thus suggests *S. barkhanus* is commensal in grayling. Grayling was suggested also as the type host for *S. barkhanus* since the infection seemed to be permanent in these fish. There is no study for the factors effecting of *S. barkhanus* microhabitat in the host. The *in vitro* culture of *S. barkhanus* from grayling done by Sterud

(1998c) was only focused on temperature-dependent growth in *S. barkhanus*, and showed that they grow at all temperature tested (5, 10, 15, and 20 °C).

S. torosa are concentrated in rectum of Atlantic cod and haddock (Poynton and Morrison 1990). It is suggested that the ileo-rectal valve may inhibit the anterior migration of the flagellates. It is also possible that cysts or trophozoites are acquired via the mouth, and pass down the gastro-intestinal tract to flourish in the favorable conditions in the rectum (Poynton and Morrison 1990).

S. vortens has been reported from ornamental fish such as angelfish and discus (Paull and Matthews 2001, Poynton et al. 1995). In addition to being an intestinal parasite in angelfish, *S. vortens* has been associated with systemic infections including hole in the head disease (Paull and Matthews 2001). It has been speculated that the hole in the head diseases represents the external manifestations of systemic infection via the gut (Paull and Matthews 2001).

S. salmonis has been reported in digestive tract of rainbow trout mostly in the pyloric region (Davis 1926, Ferguson 1979, Moore 1922a,b, Poynton et al. 2004). In rainbow trout, the concentration of diplomonads in the pyloric region is likely to be predominantly due to physiological and metabolic factors, rather than morphology, since the one valve, between intestine and rectum, is indistinct (Ezeasor and Stokoe 1980). More details of possible factors effecting microhabitat preferences for *S. salmonis* will be discussed in section 5.3. "Discussion", but an overview is given below.

Both pH and bile are suggested to play decisive roles in determining the microhabitat preference of diplomonads in trout (Buchmann and Uldal 1996, Uldal 1996). This suggestion is based on *in vitro* studies showing a pH tolerance of 5 – 10 (optimum 7.5 – 8.0), and that bile in low concentrations (30 – 960 mg / l) enhanced flagellates propagation slightly. To the best of our knowledge, there is no study on pH profile along different region of rainbow trout intestinal tract; however a range of 7.0 to 9.0 has previously been reported for salmonids

(Steffens 1989). Bile enters the pyloric region via the bile duct that passes among the pyloric caecae (Willers 1991), and its concentration is expected to decrease posteriorly.

2.4 Life cycle of *Spirotrunculus* – trophozoite and cyst

Piscine *Spirotrunculus* have a direct life cycle (needing only the fish host) with two stages; motile trophozoites in the fish, and non-motile cysts in the water. Although rigorous studies on the life cycle have not been conducted, it is assumed that trophozoites and/or cysts pass out of the fish with the faeces, spend some time in the water, and then enter a new fish via the mouth (Woo 2006). More details are given in section 1.5 “Life cycle - reproduction”.

Although both trophozoites and cysts pass in faeces (as is known for *S. salmonis* Lom and Dyková 1992, Moore 1922a,b), the cysts are resistant, and survive outside of the host, and are therefore assumed to be the stage that is primarily responsible for transmission of the infection. Piscine trophozoites do not survive for long outside of the host (only 15 – 30 minutes in *S. salmonis* (Davis 1926), and 4 hours in *S. salmonicida* (Kent et al. 1992)).

Cysts have been reported for only one of the five well-characterised species of piscine diplomonads, namely *S. salmonis* (Buchmann and Uldal 1996, Davis 1926, Moore 1922a,b, Uldal 1996). Encystment is also assumed to occur in *S. barkhanus*, *S. salmonicida*, *S. torosa*, and *S. vortens*, although this has not been demonstrated. The scarce information about cysts of piscine diplomonads may be due to the difficulties of observing them: (i) non-motile cysts are less easy to observe than the trophozoites which are easily identified by their characteristic movements even in low magnification (Kent et al. 1992, Kulda and Lom 1964, Tojo and Santamarina 1998), and (ii) cysts should also be sought in the mucus in addition to the intestinal contents, since the mucus is where they are found in *S. salmonis* in trout (Davis 1926), and in *S. meleagridis* in birds (Wilson and Slavin 1955, Wood and Smith 2005).

From our survey of the literature, it appears that the minimum infective doses (MID) of cysts essential for initiating new infection for piscine diplomonads has not been established for

any of the five well recognised species. In murine diplomonads, the minimum infective dose for a mouse is approximately 1 cyst of *S. muris* and 10 cysts of *G. muris* (Stachan and Kunsty'r 1983). In primate diplomonads, the minimum infective dose for initiating infection in humans is between 10 and 100 cysts of *G. lamblia* (Faubert 2000, Rendtorff et al. 1978).

The only report of an infective dose used for initiating piscine diplomonad infection was done in experimental studies of *S. salmonicida*, in which doses of 2×10^6 trophozoites were successfully used to initial infection by IP injection in Chinook salmon *Oncorhynchus tshawytscha* (Kent et al. 1992). However, it is difficult to extrapolate from this dose to what may be needed for the usual route of infection, which is presumed to be oral, or for the doses needed for cysts rather than trophozoites.

Limitations in our understanding of piscine diplomonad life cycles limits the scope for development of alternative treatments, which are urgently needed, since existing treatments against trophozoites are not permitted for use in food fish in Europe (more details in section 1.8 "Management and treatment of infection").

2.5 Encystment of *Spirotrunculus* – the role of flagella

In the only *in vivo* observation of encystment of *S. salmonis* in trout, Moore (1922a,b) stated that the trophozoite rounds up, decreases slightly in size, becomes quiescent, secretes a thin, hyaline cyst wall, and flagella are lost. Encystment was reported to be frequent, and assumed to be rapid. In this early description, only solitary cysts were described, there were no reports of clusters of cysts, or attachment of trophozoites to each other prior to encystment (Moore 1922a,b).

In the only *in vitro* observation of encystment of *S. salmonis* from rainbow trout, Uldal (1996) noted that "prior to encystment the trophozoites congregated in clusters adhering to each other by their flagella". This intriguing observation of flagellar attachment appears to be unique in piscine diplomonads, but unfortunately no further details were given. Recently,

clusters of cysts have been reported *in vivo* for *S. meleagridis* in ring-necked pheasants (*Phasianus colchicus*) by Wood and Smith (2005); however, it was not stated if clusters of cysts were preceded by adhesion of trophozoites. For the avian diplomonad, it was suggested that clustering of cysts provides for simultaneous ingestion of large numbers of cysts, and subsequently increase the chances of successful transmission of the parasite (Wood and Smith 2005).

The multi-functionality of flagella - for locomotion and adhesion - has been well documented in the order Kinetoplastida—the group of flagellates that include trypanosomatid parasites and bodonids. In these organisms, the flagellum is not only the classical organelle of motility, but it has also evolved to be an organelle of attachment to the invertebrate vector, playing a critical role in parasite transmission to the vertebrate host (Gull 2003).

In the following, some brief examples of multi-functionality of flagella in Kinetoplastida species are mentioned; the flagellum of the African trypanosome, *Trypanosoma brucei* is essential for cellular differentiation, and attachment to host tissue (Vaughan and Gull 2003); *Cryptobia branchialis* and *C. eilatica*, attach to gill epithelial cells, while feeding on bacteria and detritus in the surrounding water, via a contact between a ridge on the flagellar membrane and the surface of the epithelial cell (Boris et al. 2002, Diamant 1990); in the trypanoplasm-like flagellate *Jarrellia atramenti*, a commensal living in the blow hole of the pygmy sperm whale (*Kogia breviceps*), flagella attach to host material via the tip of the posterior flagellum (Poynton et al. 2001); and in the bodonid *Ichthyobodo*, flagella attach and the cytostome protrude into the host cell, and destroy the epithelial cells (Lom and Dyková 1992).

In addition to a conventional 9+2 axoneme, in the Kinetoplastida, a characteristic structure known as the paraflagellar rod (PFR), a highly ordered paracrystalline protein structure, is positioned alongside the axoneme (Gull 2003). The paraflagellar rod is a unique cytoskeletal structure that is necessary for full motility and provides support for metabolic regulators that may influence flagellar beating (Bastin et al. 1996, 2000, Kohl and Gull 1998, Landfear and Ignatushchenko 2001, Vaughan and Gull 2003).

The adhesion phenomenon in flagella has also been discovered in the green alga *Chlamydomonas*, in which flagella are not only involved in motility, but also play a key role in reproduction, by facilitating the adhesion of opposite sex gamete cells (Misamore et al. 2003, Pasquale and Goodenough 1987, Wang and Snell 2003, Yanagimachi 1994, Zhang and Snell 1993, 1994). This is the best studied system for flagellar signalling in flagellate organisms. Gametes of the green alga *Chlamydomonas* use their two flagella as sensory organelles to perceive and respond to gametes of the opposite sex in their environment (Pan and Snell, 2000). Collisions between flagella of mating-type plus (mt+) and mating-type minus (mt-) gametes allow interactions between gamete-specific flagellar adhesion molecules, the mt+ and mt- agglutinins. In the final step of fertilization, the activated gametes that had been adhering only via their flagella begin to adhere to each other via the apically localized fusion organelles on their cell bodies. This cell body adhesion is followed rapidly by cell-cell fusion and formation of a zygote.

The studies of a newly discovered cellular phenomenon termed intra-flagellar transport (IFT) have provided an inroad to learning more about flagellar signal transduction during fertilization (Pan and Snell 2002). IFT is a motility process, first discovered in *Chlamydomonas* (Kozminski et al. 1993), in which non-membrane-bound particles (IFT particles) are ferried along flagellar microtubules, from the base to the tip of the organelle, and then back (Cole 1999, Marszalek and Goldstein 2000, Rosenbaum et al. 1999). The plus-end-directed microtubule motor protein kinesin-II has been shown to be essential for movement of particles toward the tip (Cole et al. 1998, Kozminski et al. 1995, Piperno et al. 1996, Walther et al. 1994), and the cycle is completed through the action of a cytoplasmic dynein that carries IFT particles back to the cell body (Iomini et al. 2001, Pazour et al. 1998, 1999, Porter et al. 1999).

The flagellum and flagellar pocket are distinctive organelles present among all of the trypanosomatid protozoa. Currently, recognized functions for these organelles include generation of motility for the flagellum, and dedicated secretory and endocytic activities for the flagellar pocket. All vesicular traffic, both into and out of the cell, passes through the flagellar pocket and it defines the dynamic portal to host or vector environment. It is known

that some secretory products received by, and inserted into the flagellar pocket, can migrate to the flagella, and some proteins released into the flagellar pocket remain in solution (De Souza 2006).

Flagellum morphogenesis defines three membrane domains: the cell membrane, the flagellar pocket membrane, and the flagellar membrane. The flagellar and flagellar pocket membranes are component parts of the cell membrane that surrounds the entire cell. The structural and functional specialization of these two membranes has now been underscored by the identification of multiple proteins that are targeted selectively to each of these domains (Bastin et al. 2000). In the latter, the density of the glycoprotein coat is different, and it is the only place of endocytosis and exocytosis, it is the major site where cell surface receptors are located, and it is the location for the mechanisms of targeting to different domains (Bastin et al. 2000).

2.6 Pathology associated with *Spironucleus*

Spironucleus infections are usually found in the intestine and gall bladder of fish (Davis 1926, Ferguson 1979, Moore 1922a,b, Sterud et al. 1997), but can also be systemic (Kent et al. 1992, Poppe et al. 1992, Sterud et al. 1998, 2003). The intestinal and systemic infections have been associated with morbidity and mortality in aquaculture (Ferguson 1979, Kent et al. 1992, Moore 1922a,b, Poppe et al. 1992). Although diplomonad infections are commonly reported to be extra-cellular, intra-cellular infection is also observed (Davis 1926, Moore 1922a,b, Sterud et al. 2003). Most of the pathogenic *Spironucleus* infections occurred in farmed fish; there is only one recent report of a pathogenic *Hexamita*-like infection from wild Chinook salmon in Canada (Meseck et al. 2007).

Since the pathology is related to which parasite is in which host, the five well-characterised species of piscine *Spironucleus* will be discussed in view of their pathogenicities in their host as follows;

Spironucleus barkhanus

S. barkhanus was isolated from the intestine and gall bladder of wild grayling, and Arctic charr in Norway (Sterud et al. 1997, 1998). Although close to 100% of the examined fish were found to be infected, no signs of diseases were observed in fish (Sterud et al. 1997).

Spironucleus salmonicida

Spironucleus salmonicida has been recently genetically distinguished from the morphologically identical species, *S. barkhanus*, by Jørgensen and Sterud (2004, 2006). *S. salmonicida* is associated with systemic infection in farmed Arctic char and Atlantic salmon in Norway (Poppe et al. 1992, Sterud et al. 1997, 1998, 2003), and in farmed Chinook salmon in Canada (Kent et al. 1992), whereas *S. barkhanus* is found as a commensal in intestine and gall bladder of wild grayling and Arctic Charr in Norway (Sterud et al. 1997).

Systemic infection of *S. salmonicida* in farmed Atlantic salmon in Norway was characterised by sub-cutaneous and muscular boil lesions, and extensive necrotic changes in liver, kidney and spleen (Poppe et al. 1992, Sterud et al. 1997, 1998). In farmed Chinook salmon in Canada, lesions presented as whitish granulomatous nodules and inflammation in the liver and spleen, and hemorrhages of the intestinal abdominal wall (Kent et al. 1992).

Recently, wild Chinook salmon from Lake Ontario, Canada, have been affected by a Hexamita-like sp., associated with large, focal, cavernous, fluid-filled lesions in the muscle (Meseck et al. 2007). The gross pathology and histopathology suggested that the causative agent may be *S. salmonicida*, however the associated flagellate has not yet been identified by ultrastructural or molecular approaches.

Spironucleus salmonis

S. salmonis has been reported as both a commensal with no pathological findings (Allison 1963, Uzmann et al. 1965), and as a serious pathogen causing mortality in farmed trout (Davis 1926, Ferguson 1979, Moore 1922a,b, Roberts 1989, Roberts and Shepherd 1979). Therefore pathology associated with *S. salmonis* in trout varies from no effects (Uzmann et al. 1965), to a diversity of clinical signs including loss of appetite, excessive nervousness,

emaciation, pinheads, whirling sickness, and long fecal casts (Moore 1922a,b), anorexia and body weight reduction, with the presence of yellow mucus in the intestinal contents (Uldal and Buchmann 1996). Pathology of the gastro-intestinal epithelium is disputed, catarrhal enteritis was reported by Sano (1970), inflammation of the intestine was observed by Hare and Frantsi (1974), and haemorrhage in the intestine was reported by Roberts and Shepherd (1979). Hepatocellular necrosis was described by Ferguson (1979).

The relationship between host size and prevalence of infection of *S. salmonis* in rainbow trout demonstrate that diplomonad infection mostly occurs in smaller fish (70% infection in fish with standard length between 2.5-7.5 cm), which is associated with anorexia and significantly reduced weight and length (Uldal and Buchmann 1996). However, the cause of the poor growth in fingerling rainbow trout, and lack of appetite, and possible malabsorption is not yet understood.

In farmed rainbow trout infected with *S. salmonis*, Ferguson (1979) reported cytoplasmic blebbing from the intestinal mucosal cells, when studied by SEM. However, he did not observe intestinal damage to the brush border, even where the flagellates were closely apposed to the mucosal surface. He suggested a control study of the intestine to determine the significance of cytoplasmic blebbing.

To better understand the histopathology associated with diplomonad infection in rainbow trout, the anatomy and histology of the trout intestine will be given in below; the intestine of rainbow trout extends from the end of the pyloric portion of the stomach to the anus, and comprises the duodenum (pyloric region), anterior intestine, posterior intestine, and rectum (Takashima and Hibiya 1995). The duodenum has the opening of the hepatic and pancreatic ducts, and also of the pyloric caeca. The duodenum mucosa forms gentle folds, and has a histological structure which is basically the same as that of the pyloric caecae.

The histological structure of the wall of the intestine of rainbow trout consists of four basic layers, with numbers of minor layers identifiable at the light microscopic level; 1- mucosa (mucosal epithelium, lamina propria), 2- submucosa (stratum compactum, stratum

granulosum), 3- muscularis (circular muscle layer, longitudinal muscle layer) 4- serosa (tella subserosa, serous membrane) (Takashima and Hibiya 1995). The mucosal epithelium, which may come in contact with the diplomonads, is composed of a single layer of epithelial cells throughout its length, and expanded by forming folds. The apex of the cells has numerous long microvilli forming a striated border projecting towards the lumen.

Spiroucleus torosa

S. torosa has been known as commensal organism from rectum of cod and haddock in Canada (Poynton and Morrison 1990), and from rectum of saith and cod from Norway (Sterud 1998b). No clinical signs have been associated with *S. torosa* infection in fish.

Spiroucleus vortens

S. vortens infect intestine of angelfish and discus (cichlids). In addition to being an intestinal parasite, *S. vortens* has been associated with systemic infection and hole in the head disease in angelfish in Florida, USA (Poynton et al. 1995), and in discus and angelfish in the UK (Paul and Matthews 2001). *S. vortens* infection has been reported with necrotic lesions, confined chiefly to the head region, and occasionally extending posteriorly along the lateral line (Paull and Matthews 2001). In severe hole in the head disease, *S. vortens* was found in the liver, spleen, and kidney (Paull and Matthews 2001). A lip lesion is also reported in angelfish infected with *S. vortens* (Poynton et al. 1995). *S. vortens* has in addition been reported as an apparent commensal of the intestine of ide from Norway (Sterud and Poynton 2002).

Spiroucleus meleagridis* and *Spiroucleus muris

The pathology associated with *Spiroucleus* infection in non-piscine hosts was discussed more in detail in section 1.6 "Pathology", however some overview is given as follows; in young game birds infected with *S. meleagridis*, and in rodents infected with *S. muris*, there is diarrhea, depression, weight loss, high mortality, and catarrhal enteritis (Baker 1998, Barthold 1985, Cooper et al. 2004, Lloyd et al. 2005). A similar syndrome is seen in spironucleosis in Australian king parrots, which is accompanied by catarrhal enteritis or lymphoplasmacytic enteritis (Philbey et al. 2002).

2.7 Innate immunity to *Spironucleus*

Fish are the oldest animal group with an immune system showing clear similarities to the defence systems of birds and mammals. Since fish are lower vertebrates, innate immunity is more important in their defence against pathogens, than is the case in higher vertebrates, although both types of immune response are known in fish (Van Muiswinkel 1995).

Innate immunity can be considered as comprising four components: physiological barriers, anatomical barriers, humoral components, and cellular components (as has been reviewed in section 1.7 "Immunity"). Of particular relevance to enteric *Spironucleus* infection in fish are anatomical barriers, and humoral components (Woo 1996). An anatomical barrier can be for example the surface of the gastrointestinal tract, in which each of the epithelial cells maintains a close contact with its neighbours, and seals the surface of the gut, thus there is a highly dynamic structure that limits, but does not completely exclude, pathogens from entering the tissues (Macdonald and Monteleone 2005). The humoral components consist of a group of protein and non-protein components which are involved in innate defence mechanisms. Humoral components can be activated along two major routes: (i) the classical complement pathway, which is simulated by antigen-antibody immune complex; (ii) the alternative complement pathway, which is started by contact with certain microbial cell wall polysaccharides. In both cases the activation results in the lysis of pathogen cells.

The parasitic ciliate *Ichthyophthirius multifiliis* offers a useful system for the study of fish immunity against an infectious microorganism. Naive fish usually die following infection, but fish surviving parasite exposure become resistant to subsequent challenge. This resistance correlates with the presence of humoral antibodies in the serum of immune fish. Both specific and non-specific host defence mechanisms are responsible for the protection of fish against challenge infections with this ciliate. The specific humoral components comprise at least specific antibodies. The non-specific humoral elements included are the alternative complement pathway and probably lectins.

The *in vitro* plasma incubation test was described and used by Bower and Woo (1977) to study innate immunity due to the alternative complement pathway activation. The test is based on the fact that freshly collected plasma of naturally resistant fish, when incubated with the parasite, often causes significant visible damage (e. g. lysis of protozoans and cytotoxic effects on metazoans) to the parasite. *Cryptobia catastoni* was lysed under *in vitro* conditions by fresh plasma of resistant fish (rainbow trout *Onchorhynchus mykiss* from family Salmonidea, and goldfish *Carassius auratus* from family Cyprinidae), while it was active in plasma of susceptible fish (white sucker *Catostomus commerson* from family Catastomidae) (Bower and Woo 1977).

Briefly, fresh plasma was diluted serially (1:2, 1:4, 1:8, 1:16) with phosphate buffer at about 4 °C in disposable microtiter plates. A known number of parasites (20×10^6) were added to each well, incubated at about 4 °C, and examined under an inverted microscope after 3 hours. The end point was the well in which no living parasites were seen (Bower and Woo 1977).

To the best of my knowledge the *in vitro* plasma incubation test was not conducted in any piscine diplomonads, however it has been tested for mammalian diplomonads by Belosevic and Faubert (1987), when they studied the lysis and also immobilization (cytotoxicity) of *G. muris* in fresh plasma from resistant and susceptible mice. The fresh plasma was diluted serially (1:5, 1:8, 1:15, 1:30) with phosphate buffer at about 37 °C in 24-well tissue culture plates. A known number of trophozoites (1×10^5) were added to each well, incubated at about 37 °C, and examined under an inverted microscope after 1 hour. *G. muris* was lysed under *in vitro* conditions by fresh plasma of resistant mice (B10.A) while it was active in plasma of susceptible mice (A/J).

As mentioned above, the parasites are lysed via the alternative pathway of complement activation (Bower and Woo 1977). Subsequently heat inactivated plasma from resistant hosts (the plasma is incubated at 56 °C for 30 min) maybe used for parasite incubation to determine whether temperature blocked the activation of alternative complement pathway. The *in vitro* plasma incubation tests used by Bower and Woo (1977) and Belosevic and

Faubert (1987) have shown that the heat inactivated plasma from resistant host can not lyse parasites. Thus in the plasma of resistant fish that lyse the parasites are heat labile components, and the activation of complement via alternative pathway can be blocked by heat inactivation (Bower and Woo 1977).

The *in vitro* plasma incubation test suggested by Bower and Woo (1977) and Belosevic and Faubert (1987) had two major limitations; (i) the results of the plasma incubation were determined at only a single time point, which was 3 hours post incubation in the Bower and Woo (1977) study, and about 1 hour in the Belosevic and Faubert (1987) study, thus temporal changes in the condition of the parasites were not determined; and (ii) only a single parameter was recorded in Bower and Woo (1977) study, namely lysis of the parasites, thus sub-lethal effects such as cytotoxicity, indicated by abnormal movement of the parasites, were excluded.

2.8 Acquired immunity

Fish have a relatively well-developed specific immune system called acquired immunity, and are able to produce both a humoral response and a cell-mediated response to pathogens. The acquired immune system develops generally after hatching and, depending upon species and temperature, may not be fully functional until the fish is several months of age. The immune response generally takes at least 7-10 days after exposure to a parasite that the specific humoral and/or cell-mediated response can be detected in fish infected with parasites. The length of this lag phase is depended on the type and amount of antigenic stimulation, the route of exposure, ambient water temperature, age and species of fish, and the sensitivity of the immunological technique used (Van Muiswinkel 1995).

Antibodies produced in infected fish can be polyclonal, which they react with most determinants of the parasite antigens, or they are monoclonal antibodies, which react with only one antigenic determinant. Numerous techniques are used to detect and to quantify specific antibodies which are secreted into blood and /or tissue fluids in fish. Antigen-

antibody reactions may result in: (i) clumping or agglutination of antigen, (ii) precipitation of soluble antigen, and/or (iii) lysis of intact antigenic cells with complement antibodies (Forward and Woo 1996).

There are some acquired immunity studies on fishes infected by parasitic flagellates. Smith et al. (1992) have found that the tilapia, *Oreochromis aureus*, which have recovered from infection with the parasitic dinoflagellate, *Amyloodinium ocellatum*, are presumed to be resistant to re-infection. By using the ELISA test, Smith et al. (1992) detected specific antibody to the parasite.

The other example is goldfish that have recovered from *Trypanosoma danilewskyi* infections, and were protected from re-infection (Woo 1981). He suggested that acquired immunity in goldfish against the trypanosome is active while the small numbers of trypanosomes were in the blood/internal organs of recovered fish, and these continue to stimulate the immune system (Woo 1981).

Rainbow trout that had recovered from experimental infections by *Cryptobia salmositica* have also been found to resistant re-infection (Woo 1990). By using ELISA technique Woo (1990) detected specific antibodies against *C. salmositica* in rainbow trout as early as 7 days after infection.

Uldal and Buchmann (1996) found that *Spironucleus salmonis* occurs primarily in smaller fishes. In the smallest size group (fish with body lengths from 2.5 to 7.5 cm) 70% were infected, whereas only 10 and 2% were infected in the second (7.5 to 12.5 cm) and the third (12.5 to 17.5 cm) size groups, respectively (Uldal and Buchmann 1996). This study provides an evidence for a size-related infection of rainbow trout by *S. salmonis*. Whether this is caused by an inadequately developed immune system (acquired immunity) in smaller fish is unknown.

CHAPTER 3

SPECIFIC AIMS

CHAPTER 3

Specific aims

3.1 Overview

Although rainbow trout is the principal aquaculture species in Germany, and problematic diplomonad infections have often been reported (Brämick 2004, Robert and Schlotfeldt 1985, Schäperclaus et al. 1990, Schlotfeldt 1991, Sanzin 1965), there are no rigorous investigations using modern techniques. Previous studies on diplomonads from rainbow trout in Germany focused on traditional descriptive approaches, ecological parasitology, and reports of clinical signs of infection (Roberts and Schlotfeldt 1985, Sanzin 1965, Schäperclaus et al. 1990, Schlotfeldt 1991). Furthermore, despite the common occurrence of diplomonads in farmed rainbow trout in many countries, and the associated problematic infections, they are poorly understood parasites.

Our knowledge of the ultrastructural and molecular characterization of species, and factors affecting their microhabitat, is limited, thus limiting studies of epizootiology. Furthermore, transformation of trophozoites to cysts is poorly known, although this is a crucial stage in the life cycle, and may offer a new target for treatment. Quantitative approaches to pathogenesis are lacking, as is an *in vivo* method of determining susceptibility of host species, both of which restrict effective management of the infections.

To address these needs, I focussed my research on five aspects of *S. salmonis*: (1) comprehensive characterisation of the parasite using ultrastructural approaches (in collaboration with colleagues undertaking molecular characterisation), (2) investigation of pH as a factor affecting microhabitat preference of the parasite, (3) improved understanding of the life cycle and the role of flagella, via documentation of the transformation of trophozoites to cysts, (4) quantifying pathology associated with infection, and (5)

development of *in vitro* plasma incubation test to predict host susceptibility to the parasite. The results are expected to have practical benefits for aquaculture, and yield fundamental new knowledge of the cell biology of these primitive protozoans.

3.2 Characterisation of *Spironucleus*

Accurate identification of a pathogen is a key element of effective monitoring and management of disease. For diplomonad flagellates, while light microscopy is helpful in confirming the presence of diplomonad flagellates, a comprehensive approach using ultrastructure (SEM and TEM) is essential (Poynton and Sterud 2002). Recently it has been shown that the ultrastructure alone is also insufficient, since organisms that look the same may be differentiated based on sequence data from the ssu rRNA gene (Jørgensen and Sterud 2004, 2006).

There has been a lot of confusion over the true identity of the diplomonads in trout in Germany, and authors have commonly referred to them as *Octomitus intestinalis truttae* (Schmidt 1919) or *Hexamita salmonis* (Schäperclaus et al. 1990, Schlottfeldt 1991), although *Spironucleus* is probably the correct genus (Poynton et al. 2004). To the best of our knowledge, there are no published ultrastructural or molecular studies on diplomonads from German rainbow trout.

✓ Therefore the first goal of this study was to identify the genus and species of diplomonad that infects rainbow trout in Germany, and characterise the cytoskeletal and cytoplasmic characteristics, by using comprehensive scanning and transmission electron microscopy.

Furthermore, the ultrastructural study can (i) reveal insights into the functional morphology of the diplomonad cell, and (ii) recognise organelles or structures which may have potential as markers for new light microscopy specific-species diagnostic tests. Furthermore, the molecular characterisation of diplomonads is also important for what it can tell us about the earliest steps in eukaryote evolution.

The complementary sequencing of the ssu rRNA gene for the diplomonads in German rainbow trout was done by my colleagues Dr. Sterud and Dr. Jørgensen in the National Veterinary Institute in Oslo, Norway, using preserved material that I prepared and sent to them. They were able to genetically distinguish the German isolate of *Spironucleus* from other genetically identified piscine *Spironucleus* species namely *S. barkhanus* and *S. vortens* (Keeling and Doolittle 1997, Jørgensen and Sterud 2004), and *S. salmonicida* (Jørgensen and Sterud 2006). Thus the characterisation was a joint work and publication; I did not do the sequencing of *S. salmonis*, because I did not have the facilities to do it, nor did I wish to overlap the study in Norway. More information about what I did in diagnosis of *S. salmonis* is discussed in more details in section “Contributions to the papers”.

3.3 Intestinal pH and the microhabitat preference of *Spironucleus*

Knowledge of the microhabitat preference of the parasite, and determining the factors which affect this, can open new insight into the mechanisms of the interaction between the protozoan parasites and microenvironments of the intestine. Furthermore, aspects of the relationship with the host are also important for characterisation of species (Poynton and Sterud 2002).

The practical impacts of diplomonad microhabitat preference, particularly studying the role of pH as a determining factor, has important applications in fish health; such as indicating possible preferred hosts based on their intestinal pH, determining the optimum pH from *in vivo* studies - which may in turn lead to improved *in vitro* culture protocols, and finally predicting the impact of changes in pH associated with changes in diet (such as those with increased content of plant protein) on density of diplomonad infections. In recent years, there have been increasing efforts made to develop diets for carnivorous fish, such as trout, that have increasing amounts of plant protein, since fish meal production is stagnant or decreasing (Cheng et al. 2003). One can expect that such changes in diet will bring about changes in the intestinal milieu within which the diplomonads live.

The present study is the first *in vivo* study to investigate the relationship between a chemical parameter and microhabitat preference of a diplomonad in fish. I focussed on the pH, and its possible determination role on microhabitat preference of diplomonads in rainbow trout, since the importance of pH had been established in cultured *S. salmonis* by Buchmann and Uldal (1996).

The simple intestinal tract in trout comprises a short cranial ascending limb bearing pyloric caecae, and a longer caudal descending limb (intestine) extending to a distinct rectum with annulo-spiral septa arranged as a stack of caudally directed funnels (Anderson and Mitchum 1974, Ezeasor and Stokoe 1980, Smith 1989, Willers 1991). In this study, I divided the intestinal tract into four sites (pyloric, anterior, middle, and posterior regions), consistent with previous studies by Uldal and Buchmann (1996), and I determined the relationship between occurrence and density of infection, and pH in each intestinal region.

√ I approached the investigations by posing the following 8 questions: (q i) what is the occurrence of infection (presence/absence) in different intestinal regions, (q ii) what is the density of infection in different intestinal regions, and what is the relationship between density of infection in the pyloric region and that in each of the three downstream intestinal regions, (q iii) what is the pH in different intestinal regions in the intestine of uninfected fish, (q iv) what is the pH in different intestinal regions in infected fish, (q v) what is the difference between the pH in uninfected and infected fish per intestinal region, (q vi) what is the relationship between the pH and density of infection in different intestinal regions, (q vii) relationship between size of fish and infection, and (q viii) relationship between size of fish and pH.

3.4 Encystment of *Spiroucleus* – the role of flagella

The multiple functions played by flagella, including motility and attachment, are well known in parasitic kinetoplastids (Bastin et al. 2000, Kohn and Bastin 2005, Poynton et al.

2001), but little known in parasitic diplomonads. In the only *in vitro* observation of encystment of *S. salmonis* from rainbow trout, Uldal (1996) noted that “prior to encystment the trophozoites congregated in clusters adhering to each other by their flagella”, but the role of flagella was not discussed. This is consistent with a recent report of cyst clusters in *S. meleagridis* from ring-necked pheasants by Wood and Smith (2005); however, it was not stated if clusters of cysts were preceded by adhesion of trophozoite flagella.

√ To address these gaps in our knowledge, I tried to improve understanding of the life cycle and the role of the flagella, via documentation of the transformation of trophozoites to cysts.

During *in vitro* culture of the diplomonad from rainbow trout in the newborn calf serum media used by Uldal (1996), I unexpectedly observed attachment and clustering of trophozoites prior to encystment. I then performed repeated cultures of *S. salmonis* in this medium, so that I could make detailed light microscopy observations to understand the multi-functionality of the flagella. I also prepared samples for complimentary SEM studies by my colleague Dr. Sterud at the National Veterinary Institute, Oslo, Norway.

This is the first documentation of the role of the posterior flagella in encystment of a piscine diplomonad, and it may provide a new target for treatments of infection in aquaculture. Anti-adhesion therapy can be used to disturb clusters, such that smaller clusters may not meet the minimum infection doses for initiating a new infection. This is particularly important, since the effective treatment of infections using drugs such as metronidazole (Stoskopf 1993, Tojo and Santamarina 1998) is no longer licensed for use in the EU (Buchmann and Bresciani 2001).

3.5 Pathology associated with *Spirotrunculus*

Diplomonad infection in juvenile rainbow trout is commonly associated with loss of appetite, and poor weight gain (Ferguson 1979, Uldal and Buchman 1996, Woo 2006). The pathogenic mechanisms remain unknown, and rigorously controlled studies are needed

(Ferguson 1979). Investigating the histopathology of the pyloric region of the intestine in rainbow trout may find evidence to explain the loss of appetite, and poor weight gain associated with the infection.

There are few histopathology reports of the intestine of rainbow trout infected with enteric diplomonads. Uzmann et al. (1965) reported no pathology in the intestine of steelhead trout infected with enteric diplomonads. Ferguson (1979) did not observe any inflammation or necrosis of epithelial cells of pyloric caeca of upper intestine, however he found excessive mucus, and a few intra-epithelial cells (ICE) in the mucosal epithelium in the intestine of rainbow trout. He also reported a hepatocellular necrosis in liver of infected rainbow trout. There are some reports of reddening of the intestine (presumably inflammation or hemorrhage) by Roberts and Shepherd (1979), and catarrhal enteritis of epithelium of intestine by Sano (1970).

Ferguson (1979) also noted “cytoplasmic blebbing” in the intestine of infected rainbow trout by using scanning and transmission electron microscopy, where cytoplasm “leaked” into the lumen. Although he considered the cytoplasmic blebbing as an artifact, he suggested a control study. In some of my preliminary TEM sections, I also noted the cytoplasmic blebbing, and there were marked changes at the apices of the epithelial cells, with swelling, and loss of microvilli.

If enteropathy, including excess mucus, is found in the heavily infected fish showing clinical signs, but not in uninfected fish, this may indicate that malabsorption contributes to the poor weight gain.

✓ The aim was doing a control study to compare those pathological changes in the pyloric region of the intestine with *S. salmonis* in juvenile rainbow trout moderately infected with *S. salmonis* and similar fish that were uninfected. I developed a novel quantitative approach to the pathology by quantifying the following parameters in the mucosa of the pyloric region of moderately infected and uninfected fish: (i) inflammation, (ii) hemorrhage, (iii) intra-epithelial cells (IEC), (iv) the number of goblet cells per standardized length (to test for

hyperplasia), (v and vi) the length and width of goblet cells (to test for hypertrophy), (vii) cytoplasmic blebbing, (viii) swelling at tips of columnar epithelial cells, and (ix) loss of microvilli. I also examined liver sections for detecting hepatocellular necrosis.

3.6 Innate immunity to *Spirotrunculus* – *in vitro* plasma incubation test

The most effective treatment of diplomonad in fish food, metronidazole, is not permitted in Europe (Buchman and Bresciani 2001), due to serious side effects and carcinogenicity. For development of preventive strategies, a thorough knowledge of the interactions of the parasite with the piscine immune system is essential. However, there are no investigations of innate immunity to diplomonad infections in fish.

It is also important to recognise the range of hosts that a given species of diplomonad can infect, and among these, the species in which disease is likely to occur. Susceptibility to infection by piscine diplomonads is currently determined by conducting *in vivo* experimental infections (Kent et al. 1992; Uzmann et al. 1965); however this approach is expensive and time consuming. In contrast, susceptibility to infection for some other flagellates has been determined based upon the innate immunity of the host, via *in vitro* testing using an incubation of host plasma and cultured parasites (Bower and Woo 1977, Belosevic and Faubert 1987, Wehnert and Woo 1980). The study of host susceptibility can help us to predict the parasite transmission, and provide insights into possible mechanisms of innate immunity.

✓ In cooperation with Ms. Jaiwei Cheng M.Sc., I modified the *in vitro* plasma incubation test (previously used for hemoflagellates (Bower and Woo 1977)) for the enteric diplomonad found in German rainbow trout, and used the newly developed test to detect lysis and cytotoxicity of the diplomonad when exposed to plasma from susceptible and resistant fish hosts. What I did has been explained in more detail in section “Contributions to papers”.

Previously, Ms. Cheng (Cheng 2006) had successfully modified five parameters of the *in vitro* plasma incubation test used for piscine hemoflagellates (*Cryptobia*) and mammalian enteric diplomonads (*Giardia*) for piscine enteric diplomonads (*Spironucleus*).

✓ Building on that work, the aim of my study was to determine (i) whether the lysis and cytotoxicity effects can be recorded temporally, and (ii) whether the plasma from susceptible fish species has lower lytic and cytotoxic effect on the diplomonad than does plasma from resistant fish species.

CHAPTER 4

MATERIALS AND METHODS

CHAPTER 4

Materials and methods

4.1 Source of fish

Rainbow trout *Oncorhynchus mykiss* (total length: 5.5 – 22.5 cm) were collected from Seltershof farm near Berlin, Germany (12.884° E, 52.061 ° N), where they had been fed with Skretting Classic trout food (Skretting). Fish were transported alive from the farm to the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin (IGB), and subsequently held in an indoor flow-through system, supplied with tap water at 9-11 °C, and fed 3 times per day with Forellenmastfutter FM 49/22 EXSP (1.8 mm) (Aquavalent).

Two other fish species; common carp *Cyprinus carpio* (total length: 12 – 17 cm), and Atlantic sturgeon *Acipenser sturio* (total length: 117 - 128 cm) were used for conducting a plasma incubation test regarding the innate immunity and host susceptibility of fish to *S. salmonis* trophozoites. Carp and sturgeon were routinely held in a tank and an earth pond in IGB, respectively, and have not been exposed to *S. salmonis*.

4.2 Wet preparations of diplomonads

Juvenile rainbow trout were carefully held in the hand, and then struck sharply on the top of the cranium. Fry and fingerlings were decapitated by cutting just posterior to the cranium.

The body cavity was opened, and the digestive tract was removed by cutting posterior to the pharynx and anterior to the anus. The intestine was divided into four regions as suggested by Uldal & Buchmann (1996); pyloric region (ascending intestine bearing pyloric caecae),

anterior (anterior 1/3 of length from anterior descending intestine to anus, and comprising only descending intestine), middle (middle 1/3 of length from anterior descending intestine to anus, comprising descending intestine and part of rectum), and posterior (posterior 1/3 of length from descending intestine to anus, comprising rectum only).

The pyloric region of the intestine was carefully opened with fine scissors, and a sample of the contents were placed on a microscope slide and covered with a cover slip. The wet preparation was then examined with the light microscope (Olympus B061), under 100x magnification (10x objective, 10x eyepiece).

The presence of diplomonads was recorded as positive (+), and their absence as negative (-). Density was semi-quantitatively estimated as the number of flagellates under a 22×22 mm cover slip (484 mm²) (1-3 replicates per fish). I considered light infection (1+) as less than 10 flagellates/484 mm², moderate infection (2+) as 11-40 flagellates/484 mm², and heavy infection (3+) as more than 40 flagellates/484 mm² (Saghari Fard 2001).

4.3 Characterisation of *Spironucleus*

4.3.1 Ultrastructural characterisation

The fresh intestinal contents from the pyloric region of heavy infected juvenile rainbow trout were fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4), and held at 4 °C. Subsequently diplomonads were handled either *in situ* with adjacent intestinal tissue (pyloric region), or as a cell suspension, using one of two different protocols.

The post fixation of samples and electron microscopy images were routinely done by Dr. Bleiss and Ms. Marko at the Division of Molecular Parasitology, Institute of Biology, Humboldt University of Berlin. Some TEM/SEM samples were handled by Mr. Delannoy at School of Medicine, Johns Hopkins University, Baltimore, USA, and Dr. Sterud and Ms. Engeland at the National Veterinary Institute in Oslo, Norway.

***In situ* protocol (SEM and TEM)**

The pyloric region of heavily infected fish was cut into small pieces (2-3 mm³), and immediately fixed. After a maximum of one week at 4°C, post-fixation begun by centrifuging the tissue at 500 g for 5 minutes, followed by rinsing in 0.1 M Na-cacodylate buffer (pH 7.4) for 3 x 15 minutes at 4°C. The tissues were placed in 1% osmium tetroxide in Na-cacodylate buffer for 4 hours at 4 °C, then rinsed again, and transferred to 70% ethanol for subsequent processing. For SEM processing, the samples were critical point dried using CPD 030, coated with approximately 20 nm gold in a SCD 005 BAL-TEC, and viewed in LEO 1430 scanning electron microscope (done by Dr. Bleiss at Humboldt University of Berlin). For TEM, most samples were embedded in Spurr's epoxy resin, sectioned with a diamond knife using the ultra cut S (Leica, Vienna, Austria), stained with uranyl acetate followed by Reynold's lead citrate, and viewed in a Zeiss EM 900 transmission electron microscope (done by Dr. Bleiss at Humboldt University of Berlin). The other TEM samples were embedded in Eponate (Epon 812) (Ted Pella), sectioned with a Riechert Ultracut E, stained with uranyl acetate followed by lead citrate, and viewed in a Phillips CM 120 transmission electron microscope operating at 80 KV (done by Mr. Delannoy at Johns Hopkins University, USA).

Cell suspension protocol 1 (TEM)

The contents of the lumen were washed out with fixative, and held for a maximum of one week at 4°C. After centrifuging at 500 g for 10 minutes, 2% melted agarose were added to the pellet, and it was put on ice until it became solid. The gel was cut into small pieces (2-3 mm³), and rinsed in 0.1 M Hepes buffer for 2 x 10 minutes, and in 0.1 M Na-cacodylate buffer for 1 x 10 minutes. The samples were placed in 1% osmium tetroxide in 0.1 M Na-cacodylate buffer for 1 hour on ice. The gel was rinsed in distilled water for 2 x 5 minutes, and then stained in 2% aqueous uranyl acetate for 1 hour (done by Mr. Delannoy at Johns Hopkins University, USA). For TEM, the samples were then dehydrated, embedded in Eponate (Epon 812) (Ted Pella), sectioned with a Reichert Ultracut E, stained with 2% uranyl acetate (aq) for 20 min, followed by lead citrate, and viewed in a Phillips CM 120 transmission electron microscope operating at 80 KV (done by Mr. Delannoy at Johns Hopkins University, USA).

Cell suspension protocol 2 (SEM and TEM)

The pyloric region of the intestine was closed at both ends with a thread, filled with fixative via a syringe (via a portal made by cutting off one of two distinct large anterior pyloric caeca), and immersed for 3 days in fixative at 4°C. After fixation, the contents of the lumen were washed out using fixative and a 20-G syringe. The samples were then centrifuged and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hr and 15 min, and finally dehydrated in a graded ethanol series (done by Dr. Bleiss at Humboldt University of Berlin). In preparation for SEM, the diplomonads were dispersed on a Millipore filter-paper using a syringe equipped with a filter paper holder, before critical point drying and gold coating (Sterud et al. 1997). The specimens were viewed in a JEOL JSM 6400 scanning electron microscope equipped with MegaView III Soft Imaging System (done by Dr. Sterud at the National Veterinary Institute, Norway). For TEM, the samples were centrifuged to a pellet and embedded in LX 100 resin. Ultra thin sections were stained with lead citrate and uranyl acetate and viewed in a Philips CM100 transmission electron microscope equipped with MegaView III Soft Imaging System (done by Dr. Sterud at the National Veterinary Institute, Norway).

Interpretation of ultrastructure followed the guidelines of Poynton and Sterud (2002), and the recent ultrastructural description of *S. salmonis* from Irish rainbow trout (Poynton et al. 2004).

4.3.2 Molecular characterisation

The intestinal contents of two heavy infected juvenile rainbow trout were preserved in 96% ethanol. The samples were sent to my colleague Dr. Jørgenesn at National Veterinary Institute in Oslo, Norway for sequencing of the small subunit rRNA gene.

The DNA extraction was performed according to the QIAamp DNA Stool Mini Kit protocol (Qiagen). The SSU rDNA fragment from the diplomonad was amplified as described by Jørgensen and Sterud (2004). The PCR products were cloned and five positive clones were sequenced and analysed. The SSU rDNA sequence from two isolates of *S. salmonis* was

aligned using Bioedit (Hall 1999), against corresponding sequences from *S. barkhanus* (GenBank accession number AY646679), *S. salmonicida* (AY677182), *S. vortens* (U93085), *H. inflata* (L07836), *Hexamita* sp. (Z17224), *G. ardeae* (Z17210), and *O. intestinalis* (DQ366277). The resulting alignment was subjected to phylogenetic analyses using maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP). All analyses were conducted using PAUP (Swofford 2002).

For more details on materials and methods used for molecular characterisation of *S. salmonis*, please refer to Saghari Fard et al. (2007a).

4.4 Intestinal pH and the microhabitat preference of *Spironucleus*

4.4.1 Data collection

A total of 698 juvenile rainbow trout were examined for recording occurrence and semi-quantitative density of infection. The pH was recorded in 37 uninfected fish, in 21 infected fish with *S. salmonis* present in all four intestinal regions, and in 65 fish with *S. salmonis* present in only 1, 2 or 3 regions of the intestine.

The total length of fish ranged from 5.5 – 22.5 cm (mean 12.7 ± 3.5), and their weight ranged from 1.4 – 113.0 g (mean 26.8 ± 23.7); we estimate our fish were 3 – 7 months old (since the farm frequently graded the fish, no exact age was available). Although there was a large size range of fish, most (75%) were 10 – 20 cm total length (5 – 6 month old juveniles), and the mean total length ranged from 11.5 – 12.7 in four of five data sets.

Only 5% of fishes were examined the day of collection. The remaining 95% were examined within four weeks of collection, when they should have acclimated to the holding conditions in the Institute. Similar numbers of fish were examined in each of the four weeks after collection. The time interval between feeding and examination varied from 0.5 – 5.0 h.

Occurrence and density were recorded in all four intestinal regions (pyloric, anterior, middle, and posterior). The pH of the intestinal contents was recorded with an accuracy of 0.2 by using pH indicator strips (pH Indikatorstäbchen: VWR International 1.09543.0001). After the samples of intestinal contents had been taken for determining occurrence and density of diplomonads, a pH strip was laid tangentially in each of the four regions (hence four strips per fish), and gently pressed into the remaining intestinal contents. After a few minutes (allowing for maximum colour change), the strips were removed, and the pH was determined against a reference strip.

The data was collected in cooperation with Ms. Weisheit, B.Sc. student at Faculty of Agriculture and Horticulture, Humboldt University of Berlin.

4.4.2 Statistical analysis

The analysis began by using the Kolmogorov-Smirnov (K-S) test to determine whether data were normally distributed. Levene's test was used to determine homogeneity of the variance. After these preliminary tests, a series of different tests was performed for each of the 8 questions (q i – q viii) that were the specific aims of the study (see section 3.3).

The Chi-Square test (χ^2) was used to test for the differences of occurrence, and density of infection between intestinal regions (q i and q ii), (the test was carried on the number of fish, not the percentage, and for density, the proportions of infections that were light, moderate or heavy, were compared for pairs of regions). The Mann-Whitney test was used to determine the relationship between density of infection in the pyloric region and that in each of the three downstream intestinal regions (q ii). The Analysis of Variance (ANOVA) was used for the differences of pH between the intestinal regions in uninfected and infected fish (q iii and q iv, respectively), and followed by Tukey's honestly significant difference (HSD) test to compare each pair of regions. The *t*-test was used for comparing the pH in uninfected and infected fish per region (q v). The Kruskal-Wallis test (for non-normally distributed data) was used to test the differences in pH in the pyloric region between uninfected, lightly, moderately, and heavily infected fish (q vi). The Mann-Whitney test (for non-normally

distributed data) was used to test for differences in size (total length and weight) between uninfected and infected fish (q vii). Pearson's correlation coefficients were used to test the relationship between size of fish (total length and weight for 37 uninfected fish) and pH, in each intestinal region (q viii).

All statistical tests were done via SPSS (version 9), and the results were considered significant if $P < 0.05$.

4.5 Encystment – the role of *Spironucleus* flagella

4.5.1 *In vitro* culture

Two different media were used for cultivation of diplomonads from rainbow trout. The routinely used culture medium (the same as used by Buchmann and Uldal (1996)) contained minimum essential medium (SIGMA M0643), 10% heat-inactivated bovine serum (SIGMA B9433), and 29.3% sodium bicarbonate solution (7.5%w/v) (SIGMA S8761). Buchmann and Uldal (1996) used this medium to determine the optimum temperature of 10° C and optimum pH of 7.5-8.0 for diplomonad trophozoites from rainbow trout. A second culture media was also used, which differed only in the use of heat-inactivated newborn calf serum (SIGMA N4762) (the same media as used by Uldal (1996)). Uldal (1996) used this media when he observed clustering trophozoites and encystment.

To prevent microbial growth, the medium was supplemented with 2000 units penicillin/ml (SIGMA P3032), 50 µg gentamicin/ml (SIGMA G1272), and 100 units nystatin/ml (SIGMA N1638) (Buchmann and Uldal 1996, Uldal 1996). The medium was made under a laminar flow clean bench, and Rotilabo-Syring filters (ROTH P664.1) (pore size of 0.02 mm) were used to filter-sterilise the medium. The pH of the medium was regulated to 7.5 - 8.0 by using I N HCL (Uldal 1996). Media were stored at 4°C for short term use, or were kept at -20°C in the deep freeze for long term use. Deep frozen media were thawed gently at room temperature prior to use, and then kept at 10°C for inoculation of diplomonads. The pH of the thawed media was again regulated to 7.5 - 8.0.

Primary cultures were established by placing the contents of the pyloric region from 3 heavily infected rainbow trout together in approximately 45 ml of culture medium in a 50 ml plastic culture flask (ROTH CE51.1). Flask cultures were incubated vertically at 10 °C in the dark. Subcultures were made when cell density had doubled, by 1:1 dilution of culture containing diplomonads and new culture media.

4.5.2 Monitoring and fixation

The cultures were monitored for the first 30 min after inoculation, and then every day, using an inverted microscope with a 10x objective and 10x eyepiece to record the approximate density of trophozoites or the beginning of the encystment process. Upon observing flagellar attachment, clusters of flagellates, or cysts, the medium was fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) (1 volume of fixative to 1 volume of culture). The fixed cultures were held at 4°C. They were returned to room temperature and photographs were taken, using a light microscope with digital image capture.

4.5.3 Scanning electron microscopy

The fixed cultures were centrifuged and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hr and 15 min, and finally dehydrated in a graded ethanol series (done by Dr. Bleiss at Humboldt University of Berlin). In preparation for SEM, the diplomonads were dispersed on a millipore filter-paper using a syringe equipped with a filter paper holder, before critical point drying and gold coating (Sterud et al 1997). The specimens were viewed in a JEOL JSM 6400 scanning electron microscope equipped with MegaView III Soft Imaging System. Since this millipore filter technique was not available in Humboldt University of Berlin, this part was done by Dr. Sterud and Ms. Engeland at the National Veterinary Institute in Oslo, Norway.

4.6 Pathology associated with *Spironucleus*

4.6.1 Overview

The aims of this study were to develop a standard quantitative approach to the pathology, and determine the relationship between the infection and enteropathy. I looked for the following pathology parameters, which have previously been reported from the intestine of *S. salmonis* infected rainbow trout; inflammation, hemorrhage, intra-epithelial cells (IEC), excess mucus (by counting the number and measuring the size of goblet cells), cytoplasmic blebbing; and the two parameters I found from the preliminary TEM; swelling at tips of columnar epithelial cells, and loss of microvilli. I also checked liver sections for evidence of hepatocellular necrosis.

4.6.2 Harvesting of tissues

A total of 15 uninfected fish, and 15 moderately infected fish were examined for the histopathological study. The fish were matched for total length, with the range and the mean in each group being: uninfected fish 10.8-17.9 cm (mean 12.5 cm), and infected fish 10.8-17.5 cm (mean 12.6 cm); these lengths were not significantly different (unpaired *t*-test). It was difficult to match for age, since the farm frequently graded the fish, and no exact age was available.

The pyloric region of the intestine was carefully opened with fine scissors (only the first 2-3 mm), and a sample of the contents were placed on a microscope slide and covered with a cover slip. The wet preparation and the recording of occurrence and density of infection in the pyloric region of the intestine were explained in section 4.2 "Wet preparation of diplomonads".

If fish were infected moderately, the digestive tract including the intestine, was removed from the body cavity, and put on a petri dish on ice, in order to minimize deterioration of the tissue during harvesting of diplomonads and the intestinal samples. The cold (4 °C) 10 %

formalin (diluted in PBS) was slowly injected into the intestinal lumen from approximately 1 cm posterior to the pyloric region.

The pyloric region was cut transversely in two parts; the anterior part of pyloric region “ant”, which is close to the stomach and is completely covered with pyloric caecae, and the posterior part of pyloric region “post” which is close to the upper intestine and is partly covered with pyloric caecae. The posterior part was chosen for this study because it was not damaged during the opening of the anterior part of the pyloric region for collection of digesta for determining density of infection.

The liver was also removed, laid out on the side, and was cut into 3 equal pieces transversely. A razor blade was used to trim the tissues, in order to prevent any physical damage to the tissues. The tissues (the “post” part of the pyloric region, and 3 pieces of liver) were fixed in cold (4 °C) 10 % formalin in glass tubes.

4.6.3 Histology

The “post” part of the pyloric region, and the 3 pieces of liver from each fish were placed in one cassette (the intestine oriented so that transverse sections would be made), and embedded in paraffin. The embedded tissues were sectioned at 3 µm. For each fish, 2 sections were stained with hematoxylin-eosin (H&E) for an overview of the histology of the intestine and liver, and 2 sections were stained with periodic acid-schiff (PAS) combined with alcian blue (AB) for demonstrating the acidic (deep blue) and neutral (red) goblet cells in the intestinal mucosa.

4.6.4 Pathology quantification

The histological sections of pyloric region of the intestine and liver were initially examined at 200x magnification to find sections of the intestine that were in the required transverse section. Sections from more than 15 uninfected and infected fish had been prepared, so that

samples from fish with sub-optimally oriented tissues could be discarded, allowing the required number of fish to be examined.

Whole transverse section of pyloric region (H&E)

Each slide was examined at 400x magnification for recording of 4 pathology parameters in the mucosa of the pyloric region: inflammation (presence/absence, severity), hemorrhage (presence/absence, severity), intra-epithelial cells (IEC) (number per entire cross section), and cytoplasmic blebbing (presence/absence, % affected), and at 600x magnification for recording 2 pathology parameters: swelling at tips of columnar epithelial cells (presence/absence, % affected), and loss of microvilli (presence/absence, % affected). At 400x magnification, I also examined liver sections for hepatocellular necrosis (presence/absence, severity).

Other pathology signs in the intestine (not previously reported as associated with *S. salmonis*) were also recorded as present/absent, and if present, the % of the cross section affected was noted (estimated to the nearest 5%). The data were recorded in Table 4.1 (Overview of histopathology).

Detailed examination of folds in pyloric region (AB/PAS)

Combined periodic acid-schiff (PAS) and alcian blue (AB) sections, one per fish, were examined at 600x magnification, for study of the goblet cells. In each section, five individual folds were selected for study, the folds were long and projecting into the lumen perpendicularly to the sub mucosa (Fig. 4.1).

Table 4.1. Overview of histopathology in infected rainbow trout by *S. salmonis* - entire transverse section, (H&E) – quantitative study (3 μ , 400x / 600x)

Date exam	Fish no.	Infect/uninf	Weight	Length	Inflammation	Haemorrhage	IEC

....continue	Cytoplasm blebbing	Swelling at tip epithelium cells	Loss of microvilli	Liver	Remarks (strange folds)

- Inflam + hemorr + liver (hepatocellular necrosis) : presence/absence mild, moderate, severe
- IEC : number per entire section
- cytoplasm bleb + swelling tip epithelium + loss microvilli : presence/absence, % affected



Fig. 4.1. The transverse section of the pyloric region from the intestine of uninfected rainbow trout, 200x magnification. The arrows show the 3 examples of chosen folds for further study on the number and size of goblet cells. Scale bar = 40 μm .

The length of each fold was measured at the level of the 200x magnification. The folds were considered as an inverted U-shape, and the fold length was measured from the top of the fold to the line that joint the bottom of the mucosal epithelium on the left to the bottom of the mucosal epithelium on the right (Fig. 4.2).



Fig. 4.2. The transverse section of folds in the pyloric region from the intestine of uninfected rainbow trout, 200x magnifications. Note measuring the length of a fold, (yellow line). Scale bar = 40 μm .

To determine if there was hyperplasia (increase in cell numbers) of the goblet cells in infected fish, the number of goblet cells per 100 μm of fold length was compared in uninfected and infected fish. For each fold, the number of goblet cells was recorded along the fold, beginning at the bottom of the fold (the bottom of the mucosal epithelium) on the left, passing up the fold to the tip, and then down the fold to the bottom on the right (the bottom of the mucosal epithelium). The number of goblet cells was recorded in three categories: (i)

complete, with typical goblet profile comprising narrow stem and broad bowl at the mucosal surface; (ii) putative, only a small indistinct profile, staining consistent with a typical goblet cell, these were most common at the base of the folds; and (iii) all, namely the sum of the complete and the putative goblet cells (Fig. 4.3). The mean number of goblet cells per 100 μm of fold/fish was determined by calculating the mean of the five individual folds.

To determine if there was hypertrophy (increase in cell size) of the goblet cells in infected fish, the length and width of goblet cells were compared in uninfected and infected fish. The dimensions were recorded for the three largest goblet cells in each of the five folds, thus a total of 15 goblet cells were measured for each fish, and their mean dimensions were calculated for each fish. Since the goblet cells have two parts: the broad part, and the narrow base, I only measured the length and width of the broad goblet part at the mucosal surface, since this was the easiest to see (Fig. 4.3).



Fig. 4.3. The transverse section of a fold in the pyloric region from the intestine of uninfected rainbow trout, 400x magnification. Note measuring the length (yellow) and width (orange) of a “complete” goblet cell. The red arrow shows a “putative” goblet cell. Scale bar = 20 μm .

I was not able to easily and reliably distinguish between acidic and neutral contents of the goblet cells, since the expected distinct staining characteristics, namely acidic as deep blue, and neutral as red, were rarely seen. In most cases the goblet cells stained blue / mauve, suggesting they were predominantly acidic. The data were recorded in Table 4.2 (Detailed histopathology).

Table 4.2. Detail of histopathology in infected rainbow trout by *S. salmonis* – five folds per fish, (PAS/AB) – quantitative study (3 μ , 40x)

Date of exam	Fish no.	Infection	Weigth	Length	Fold no.	Fold length
					1		
					2		
					3		
					4		
					5		
					mean		

Compl. goblet cell	Putative goblet cell	All goblet cell	Length big goblet 1 (L 1)	Width Big goblet 1 (W 1)	L 2	W 2	L 3	W 3

Liver (H&E)

Each slide was examined at 400x magnification for recording of the presence or absence of hepatocellular necrosis, and if present, the severity of the necrosis was noted. The data were recorded in Table 4.1 (Overview of histopathology).

4.6.5 Statistical analysis

The quantitative data available for each pathological parameter from infected and uninfected fish were statistically analysed for a possible significant differences. For hyperplasia (the number of goblet cells per 100 μm of fold length), the data were tested by unpaired *t*-test for “all” goblet cells (complete plus putative), and for “complete” goblet cells. For hypertrophy (the size of goblet cells), the data were tested using unpaired *t*-test for the length and width of goblet cells.

For each of the other pathologies, (vacuolisation, clubbing, loss of fold architecture, shortened folds, and crowded nuclei), the proportion of *S. salmonis* infected and uninfected fish was tested Fishers exact test, and the % affected in the section was tested by Analysis of Variance (ANOVA).

All statistical tests were considered significant if $P < 0.05$.

4.7 Innate immunity to *Spironucleus* – *in vitro* plasma incubation test

4.7.1 Experimental design

The experiment aimed to develop and apply the *in vitro* plasma incubation test previously used in haemoflagellates and *Giardia* (Belosevic and Faubert 1987, Bower and Woo 1977, Wehnert and Woo 1980) to the enteric piscine diplomonad, *S. salmonis*. The goal was to use the test to predict host susceptibility to infection, by determining the effect of plasma of resistant fish (carp and sturgeon) and susceptible fish (rainbow trout) on trophozoites of *S. salmonis* from German rainbow trout.

The basic premise of the test was as follows; the wells were inoculated with culture medium, and fresh plasma in serial dilutions, followed by known numbers of cultured trophozoites. Control wells lacked plasma. Well plates were held at 10 °C in the dark, and monitored at several time points post-inoculation for evidence of lysis and cytotoxicity.

This experimental study was done in cooperation with the research study of Ms. Cheng, M.Sc. student at Faculty of Agriculture and Horticulture, Humboldt University of Berlin. Ms. Cheng did much of the work on protocol development, and I focussed on the lysis and cytotoxicity time points and testing plasma of different fish species (more details in section “Contributions on papers”).

4.7.2 Developing *in vitro* plasma incubation test – refining the protocol

In order to develop a successful test, a combination of parameters had to be tried, in order to find the optimal combination of techniques. The optimal conditions of 5 parameters - culture media, collection of blood, incubation temperature, plasma preparation and dilution solution, and type of well plate – were known in advance of my research (Cheng 2006).

For the remaining 7 parameters – density of trophozoites in the well, plasma preparation and dilution solution, serial dilution, time check points, detecting lysis and cytotoxicity, control, and replications, optimal conditions had to be determined during protocol development.

Parameters known in advance

(i) Culture media for maintaining stock cultures of *S. salmonis*: The culture medium contained minimum essential medium (SIGMA M0643), 10% heat-inactivated bovine serum newborn calf serum (SIGMA B9433), and 29.3% sodium bicarbonate solution (7.5%w/v) (SIGMA S8761), as used by Buchmann and Uldal (1996). The antibiotics used to prevent microbial growth, and the regulating the pH of culture medium were discussed in detail in section 4.5.1 “*In vitro* culture”.

(ii) Collecting blood: Fish blood was collected by heparinized syringes. Each syringe was prepared by rinsing (by filling and emptying) with a heparin solution (ROTH 7692.1), and allowed to air dry for 24 - 48 h (Roberts 1989). The fish was held and the blood was collected from the caudal vein of each fish, and was transferred to 2 ml Ependorf tubes. The minimum of 0.5 ml blood was needed for further process.

(iii) Temperature: The previous *in vitro* plasma incubation test were done in different environmental temperature: at 4 °C for *Cryptobia* and *Trypanoplasma* (Bower and Woo 1977, Wehnert and Woo 1980), and at 37 °C for *Giardia* (Belosevic and Faubert 1987). However, the optimal *in vitro* culture temperature for diplomonads from rainbow trout was reported 10 °C (Buchmann and Uldal 1996). Therefore insulated boxes at 10 °C were set up in which the plates were incubated during the plasma incubation test in the present study. I used a styfoam boxes with ice, and checked temperature of box to keep it at 10 °C.

(iv) Plasma preparation and dilution solution: Since centrifuging the blood at high speed (13,000 g) and for a long time (10 min) may damage the blood cells, blood samples were centrifuged at 3,000 g for 5 min with decanting and re-centrifugation of the supernatant for another 5 min. The collected plasma was kept at 4°C and used as fresh. For making a serial dilution of plasma, the culture medium was used, and trophozoites were observed better in the red-pink colour of the medium than in the transparent PBS under the inverted microscope.

(v) Well plate: In order to determine the most efficient observation of parasites (number and movement), two well plates had been compared; 96 well plate (circular well, 382 µl, 32 mm²) (BIOUSING 0010708), and 384 well plate (square well, 138 µl, 11 mm²) (GRINER BIO-ONE 781182). Both well plates are transparent, surface treated, sterile, and flat bottomed.

The 384 well plate was more suitable than 96 well plate, because the square area of the well could be observed under the inverted microscope in one field of view at 10x magnification, whereas in 96 well plate, which only 1/4 area of the well could observe in the same magnification.

Parameters optimised during my experiments

(vi) Density of trophozoites in the well: It was important to determine the suitable density of trophozoites, and volume of liquid in the well. If there were too many trophozoites in the well, counting them was very difficult. If there were too few, it could be difficult to have large enough numbers upon which statistical tests could be conducted. Therefore, three ranges of trophozoites (10-20, 20-40, and 40-50 trophozoites per well) were tested in different volume of culture media (40, 50, and 100 μ l).

The desired range of density of trophozoites in culture was arranged by adding new culture medium to the solution.

(vii) Plasma – serial dilutions: Different serial dilutions from 1:4 to 1:2048 were tested for all three fish species; rainbow trout, carp, and sturgeon, to find out the appropriate range of serial dilutions of plasma (e.g. testing which plasma concentration has similar affect as the control with no plasma).

(viii) Time check points: In previous studies, the time check points were 3 hours after inoculation (Bower and Woo 1977, Wehnert and Woo 1980), or 5, 10, 20, and 30 min after inoculation (Belosevic and Faubert 1987). In the present study, in order to monitor temporal changes in lysis and cytotoxicity, the effects of different plasma concentrations on the diplomonad were monitored at 4 time check points post- inoculation: 5, 30, 60, 120 min.

(ix) Detecting lysis and cytotoxicity: The lysis was determined by differences between the number of trophozoites in the control and with the numbers in each time check points. For detecting cytotoxicity, the behaviour and movement of trophozoites in different plasma concentration were monitored.

(x) Control: The control wells were the ones with trophozoites but without plasma.

(xi) Replications: In previous *in vitro* plasma incubation studies, the investigations were done in 2-11 replicates for haemoflagellates (Bower and Woo 1977, Wehnert and Woo 1980), and 5

replicates for *Giardia* (Belosevic and Faubert 1987). In the present study, the experiment was conducted three times for each fish with three control wells in each experiment to see the variations of data.

4.7.3 Performing *in vitro* plasma incubation test

Based on the result of the parameters which are tested for developing *in vitro* plasma incubation test for use on enteric piscine diplomonad *Spironucleus*, I could perform the modified test on *S. salmonis* from rainbow trout. I used rainbow trout as susceptible host, and common carp and Atlantic sturgeon as resistant hosts. Rainbow trout was exposed to *S. salmonis*, but carp and sturgeon were believed never to have been exposed to *S. salmonis*. The common carp in general can have enteric diplomonads (though probably another *Spironucleus* species, not *S. salmonis*) (Wu et al. 2003), and Atlantic sturgeon, not known as a host for diplomonads, although other *Acipenser* species can be infected with diplomonads in their gall bladder (Bauer et al. 2002).

The primary plan was to test at least 10 individual fishes for each species. However due to the difficulties of *in vitro* culture and limited parasite resources, only 3 rainbow trout (normal host for *S. salmonis*), 2 common carp (susceptible host) and 3 Atlantic sturgeons (resistant host) were tested. Two species were always tested in parallel in each experimental day, of which at least one was a rainbow trout.

CHAPTER 5

RESULTS AND DISCUSSIONS

CHAPTER 5

Results and discussions

5.1 Characterisation of *Spirotrunculus salmonis*

5.1.1 Result: Surface morphology

The flagellates were pyriform, with a posterior end that was more or less tapered, and bore a caudal projection; the surface of the body was unadorned (Fig. 5.1.1). In some flagellates, the surface was not completely smooth, with some rounded swellings reaching about 0.3 μm in diameter (Fig. 5.1.1b), and discharging vacuoles reaching about 0.4 μm in diameter (Fig. 5.1.1c). The recurrent flagella emerged from the body on both sides of the caudal projection (Fig. 5.1.1d,e).

5.1.2 Result: Internal structure - identification to genus

The 2 elongate nuclei tapered anteriorly and were multi-lobed and intertwined apically (Fig. 5.1.2a–c). The 2 recurrent flagella passed posteriorly between the 2 nuclei (Fig. 5.1.2d), and each recurrent flagellum was surrounded by a flagellar pocket. Kinetosomes (syn. Basal bodies) lay anterior-medial to the nuclei at the apex of the cell (Fig. 5.1.2e). Kinetosomes of 2 anterior flagella (k1, k3) and 1 recurrent flagellum (kr) were close to each other, and made a triangle form (Fig. 5.1.2f). There was a right angle between k1 and kr, and k3 lay between at an angle of 45° to both (Fig. 5.1.2f).

5.1.3 Result: Internal structure - identification to species (cytoskeleton)

Supra-nuclear microtubular bands extended over the anterior of the nuclei, closely following the nuclear membranes (Fig. 5.1.2e). Infra-nuclear microtubular bands ran along the medial

surface of the nuclei to the opening of the striated lamina surrounding the recurrent flagella (Fig. 5.2d). Direct microtubule bands radiated from the opening of the striated lamina (Fig. 5.1.2d).

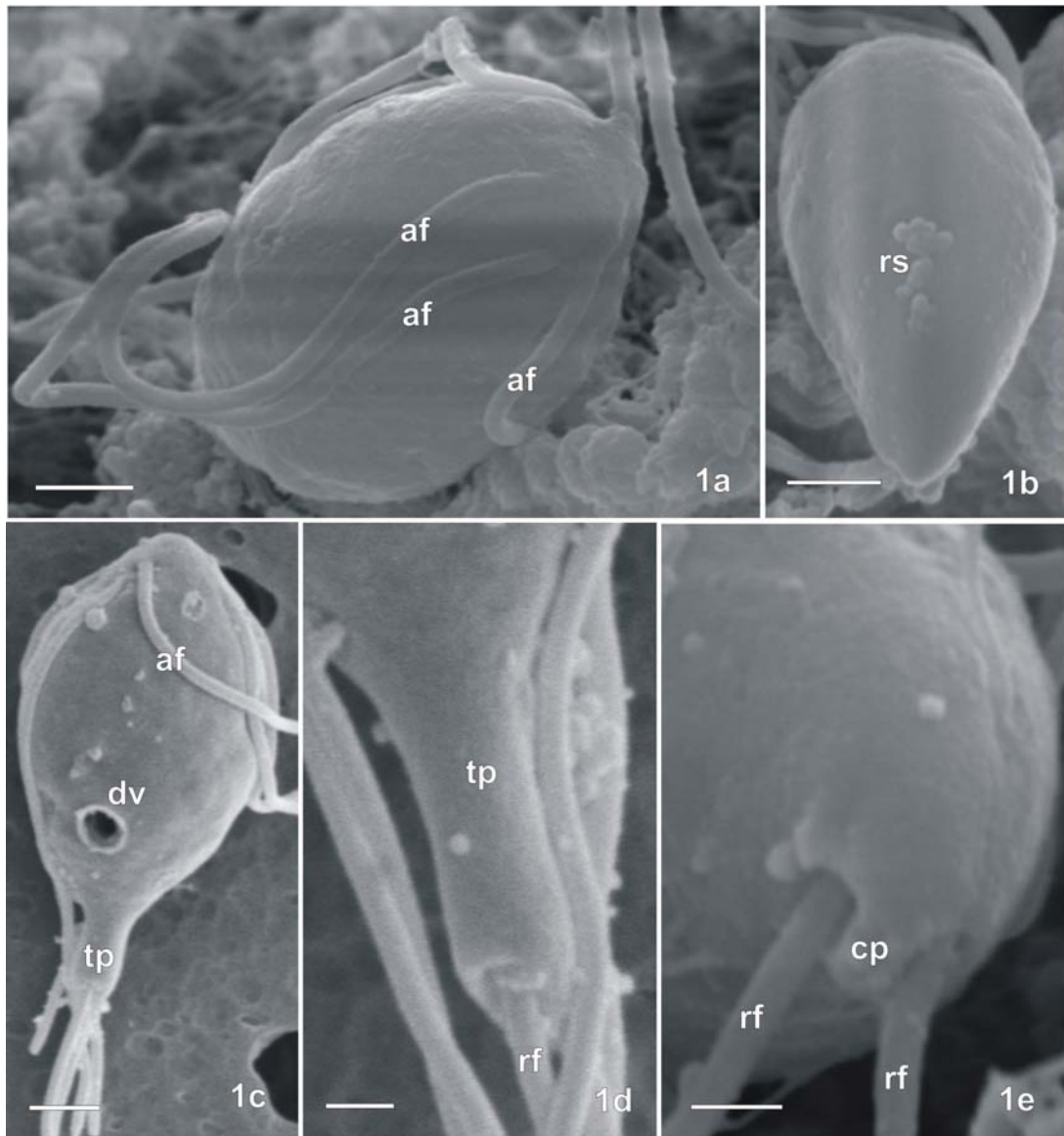


Fig. 5.1.1. *Spironucleus salmonis* infecting *Oncorhynchus mykiss*. Scanning electron micrographs of diplomonad flagellate from intestine of fingerling rainbow trout showing surface architecture. (a) Anterior-lateral view of flagellate showing unadorned surface, with set of 3 anterior flagella (af). (b) Dorsal or ventral view of flagellate showing rounded swellings (rs). (c) Lateral view of flagellate showing anterior flagella (af), tapered posterior end (tp), and discharging vacuole (dv). (d) Lateral view of flagellate showing tapered posterior end (tp), and recurrent flagella (rf). (e) Lateral view of flagellate showing conical process (cp) and recurrent flagella (rf).

and an emerging recurrent flagellum (rf). (e) Posterior end of flagellate showing recurrent flagella (rf) that emerged from body on both sides of caudal projection (cp). Scale bars = (a,b) 1 μm , (c) 2 μm , (d,e) 0.5 μm . Reproduced with permission from Diseases of Aquatic Organisms.

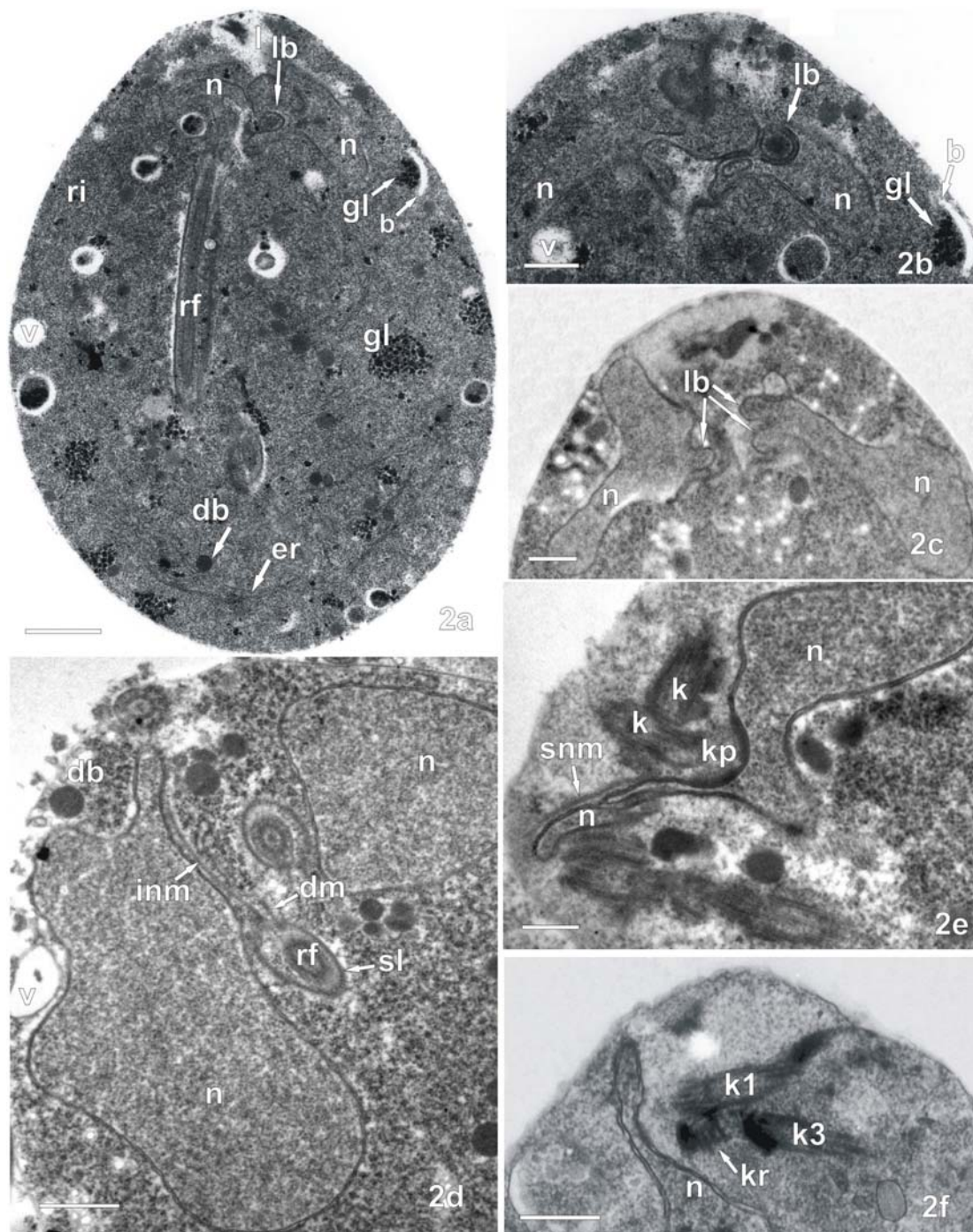


Fig. 5.1.2. *Spironucleus salmonis* infecting *Oncorhynchus mykiss*. Transmission electron micrographs of diplomonad flagellate from intestine of fingerling rainbow trout showing features for identification to genus. (a) Longitudinal section through cell showing the 2

elongate nuclei (n) that are S-shaped, and multi-lobed (lb); note recurrent flagellum (rf), light-staining homogenous cytoplasm (l) in the apex, numerous free ribosomes (ri), bowl-shaped membranous structure (b), aggregation of glycogen (gl), endoplasmic reticulum (er), electron-dense bodies (db), and vacuoles (v). (b) Longitudinal section through the apex of cell showing that the 2 nuclei (n) are multi-lobed (lb), and intertwined apically; note bowl-shaped membranous structure (b) with closely oppressed aggregation of glycogen (gl), and vacuoles (v). (c) Longitudinal section through apex of cell showing that the 2 nuclei (n) are multi-lobed (lb) and intertwined apically. (d) Transverse section through anterior of cell showing the 2 recurrent flagella (rf) pass posteriorly between the 2 nuclei (n); infra-nuclear microtubular bands (inm) run along medial surface of nuclei to opening of striated lamina (sl) surrounding the recurrent flagella; direct microtubular bands (dm) radiate from opening of striated lamina; note electron-dense bodies (db) and vacuoles (v). (e) Oblique section through kinetosomes (k) lying anterior-medial to nuclei (n) at apex of cell; the base of a kinetosome lies in the cup-shaped kinetosomal pocket (kp); note supra-nuclear microtubular bands (snm) extended over anterior of nuclei, closely following nuclear membranes. (f) Oblique section through kinetosomes of the 2 anterior flagella (k1, k3), and 1 recurrent flagellum (kr) close to a nucleus (n) at apex of cell; note right angle between k1 and kr and that k3 lies between them at an angle of 45° to both. Scale bars = (a) 1 μm , (b,c,d,f) 0.5 μm , (e) 0.25 μm . Reproduced with permission from Diseases of Aquatic Organisms.

In transverse section through the middle of the cell, posterior to the nuclei, the 3 microtubular bands accompanying the recurrent flagella radiated at the opening of striated lamina (Fig. 5.1.3a,b). The radiate pattern of microtubular bands comprised (from left to right in Fig. 5.1.3a,b) an undulating row of 4 microtubules lying between the tip of the striated lamina and extending into the opening of the striated lamina (direct band), a straight row of 3 microtubules radiating away from the opening of the striated lamina (direct band), and a curved row of 7 microtubules extending over the distended side of the striated lamina (infra-nuclear band) (Fig. 5.1.3a,b). The flagellar pocket was surrounded by an asymmetrical U-shaped striated lamina when viewed in transverse section (gutter-shaped in 3 dimensions) (Fig. 5.1.3a,b). At the extreme posterior end of cell, the U-shaped striated lamina was expanded when viewed in transverse section (Fig. 5.1.3c).

Electron-dense plaques were visible at the anterior part of the cell (Fig. 5.1.3d). An electron-dense plaque lay adjacent to anterior kinetosome, and another lay just posterior to the basal portion of the recurrent flagella, between the axoneme and the striated lamina (Fig. 5.1.3d). These dense plaques are distinguished from dense bodies by their precise position in the cytoplasm, their darkly staining structure, and their size and shape (as described by Poynton et al. (2004) for *Spironucleus salmonis* from rainbow trout in Ireland).

5.1.4 Result: Internal structure - identification to species (cytoplasm)

The cytoplasm of the flagellate had a light-staining homogenous region in the apex (Fig. 5.1.4a), and an organelle-rich heterogeneous region in the rest of the cell (Fig. 5.1.2a). Heterogeneous cytoplasm contained numerous free ribosomes, bowl-shaped membranous structures, aggregations of glycogen, endoplasmic reticulum, electron-dense bodies, and vacuoles (Figs. 5.1.2a,b and 5.1.4a). The aggregations of glycogen were present in at least 3 distinct locations, i.e. some glycogen was irregularly scattered throughout cytoplasm (Fig. 5.1.2a), some glycogen lay within the bowl-shaped membraneous structures (Fig. 5.1.4a), and some glycogen was distributed longitudinally between the flagellar pocket and striated lamina (Fig. 5.1.4a). Endoplasmic reticulum was distributed irregularly in the cytoplasm and around the recurrent flagella (Figs. 5.1.2a and 5.1.4c). One membrane-bound electron-dense body was extended adjacent to endoplasmic reticulum, and appeared as 3 interconnected dense bodies (Fig. 5.1.4b); another was elongate in section (Fig. 5.1.4c). Some electron-dense bodies were completely membrane bound (Fig. 5.1.4d), some had the same high contrast material at the periphery (Fig. 5.1.4e), while others did not appear to have a membrane (Fig. 5.1.4d). A discharged vacuole was visible just beneath the cell membrane (Fig. 5.1.4f).

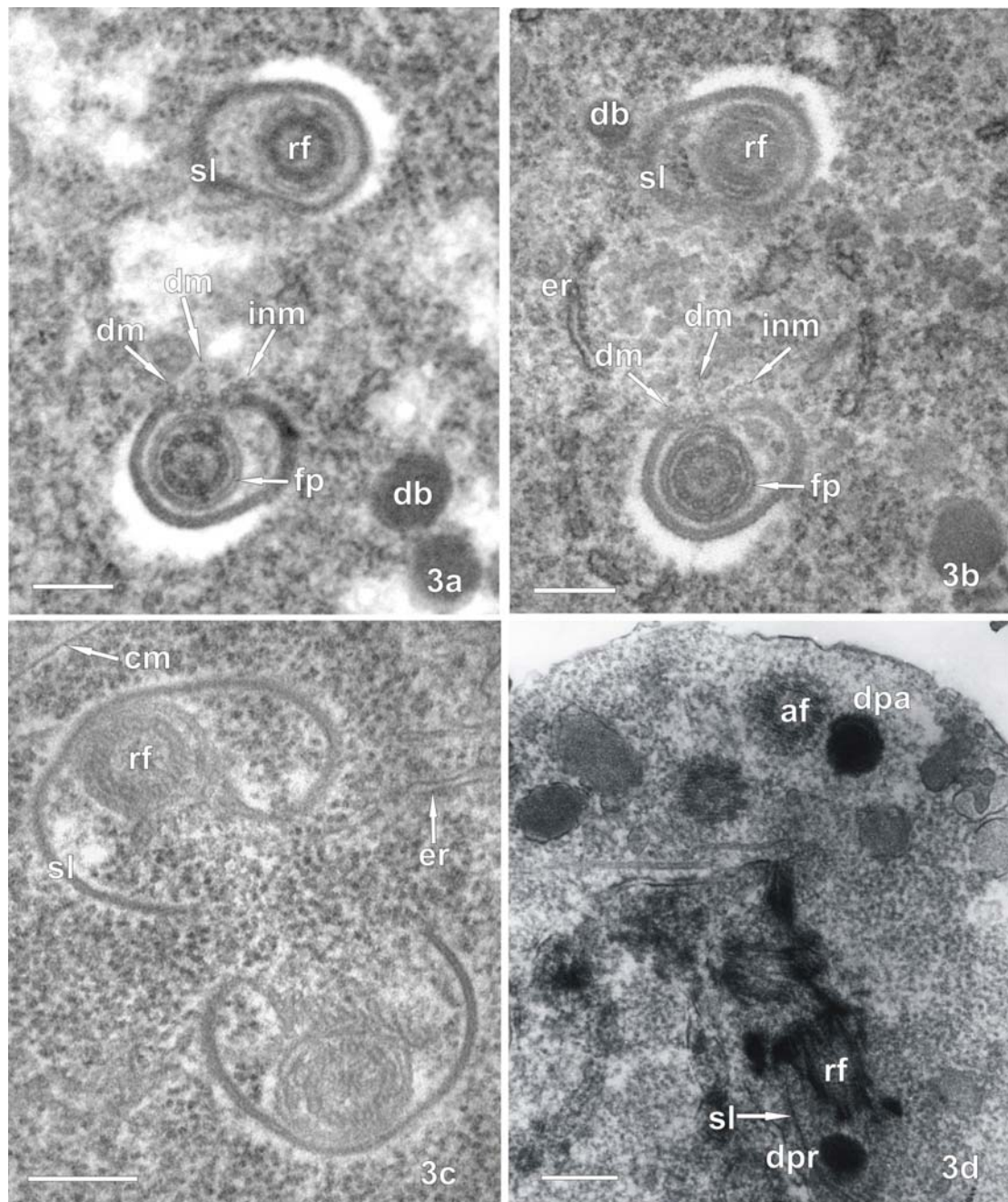


Fig. 5.1.3. *Spironucleus salmonis* infecting *Oncorhynchus mykiss*. Transmission electron micrographs of diplomonad flagellate from intestine of fingerling rainbow trout showing cytoskeletal features for identification to species. (a) Transverse section through middle of cell, posterior to nuclei, showing radiated pattern of 3 microtubular bands at the opening of striated lamina (sl) surrounding flagellar pocket (fp) and recurrent flagellum (rf). The 3 microtubular bands comprise (from left to right): undulating row of 4 direct microtubules (dm) lying between tip of striated lamina and extending into the opening of the striated lamina; straight row of 3 direct microtubules (dm) radiating away from opening of striated

lamina; and curved row of 4 infra-nuclear microtubules (inm) extending over distended side of striated lamina; note electron-dense bodies (db). (b) Transverse section through middle of the cell, posterior to nuclei, showing radiated pattern of 3 microtubular bands at opening of striated lamina (sl) surrounding flagellar pocket (fp) and recurrent flagellum (rf), undulating row of 4 direct microtubules (dm) lying between tip of striated lamina and extending into opening of the striated lamina, straight row of 3 direct microtubules (dm) radiating away from opening of striated lamina, and curved row of 7 infra-nuclear microtubules (inm) extending over distended side of striated lamina; note the endoplasmic reticulum (er) around the 2 recurrent flagella, and electron-dense bodies (db). (c) Transverse section through the 2 recurrent flagella (rf), lying close to each other at posterior end of cell; asymmetrical U-shaped striated lamina (sl) is expanded; note adjacent cell membrane (cm). (d) Oblique section through anterior part of cell; an electron-dense plaque (dpa) lies adjacent to an anterior flagellum (af), and another electron-dense plaque (dpr) lies posterior to basal portion of the recurrent flagellum (rf), between the axoneme and striated lamina (sl); the dense plaques in this figure are circular (a shape also consistent with dense bodies) because of the oblique section; however, note the precise position of 'dpa' and 'dpr', and their darkly staining structure, which confirms these structures as dense plaques. All scale bars = 0.25 μm . Reproduced with permission from Diseases of Aquatic Organisms.

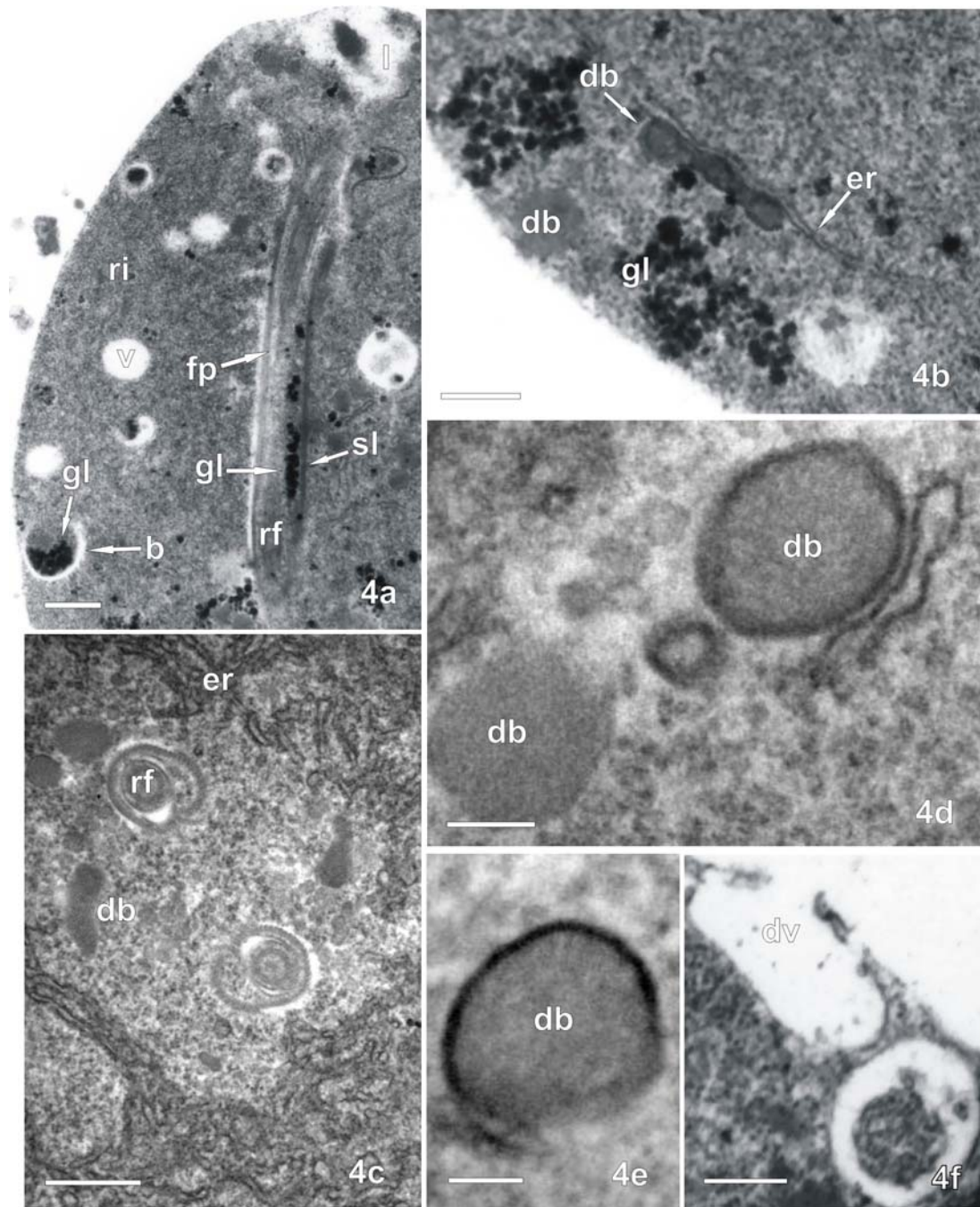


Fig. 5.1.4. *Spironucleus salmonis* infecting *Oncorhynchus mykiss*. Transmission electron micrographs of diplomonad flagellate from intestine of fingerling rainbow trout showing cytoplasmic features for identification to species. (a) Longitudinal section through body showing light-staining homogenous region (l) in apex, and a recurrent flagellum (rf) passing through middle of cell. Some glycogen (gl) is irregularly scattered throughout cytoplasm; some glycogen is distributed longitudinally between the flagellar pocket (fp) and striated lamina (sl), and some glycogen lie within bowl-shaped membranous structures (b); note

numerous free ribosome (ri), and vacuoles (v). (b) Longitudinal section through body showing one membrane-bound electron-dense body (db) which is extended adjacent to endoplasmic reticulum (er) and appears as 3 interconnected dense bodies; note aggregations of glycogen (gl), and non membrane-bound electron-dense body (db). (c) Transverse section through middle of cell showing 2 recurrent flagella (rf) surrounded by irregularly distributed endoplasmic reticulum (er); note also electron-dense bodies (db), one of which is elongate. (d) One electron-dense body (db) that is completely membrane bound (right), and another electron-dense body that does not appear to have a membrane (left). (e) Electron-dense body (db) with high contrast material at the periphery. (f) Discharged vacuole (dv) beneath cell membrane. Scale bars = (a,c) 0.5 μm , (b,f) 0.25 μm , (d,e) 0.125 μm . Reproduced with permission from Diseases of Aquatic Organisms.

5.1.5 Result: Molecular characterisation

The molecular characterisation and phylogeny analysis has been carried out by Dr. Anders Jørgenson and Dr. Erik Sterud at National Veterinary Institute in Oslo, Norway.

An approximately 1400 bp fragment was amplified from diplomonads from 2 rainbow trout and cloned. The pair-wise variations between the 5 sequenced clones from 1 fish comprised on average 4 out of 1405 positions. The pair-wise variations between clones from 2 individual fish also comprised on average 4. Two consensus sequences were constructed from the 5 clones obtained from each fish: S.s-1 (Accession No. DQ394703) and S.s-2 (Accession No. DQ394704). Based on an alignment of ssu rDNA from 2 isolates of *Spironucleus salmonis* from rainbow trout against *S. barkhanus* from wild Arctic charr, *S. salmonicida* from farmed Atlantic salmon, and *S. vortens* from angelfish, pair-wise similarities were calculated for 1441 positions (see Fig. 5 in Saghari Fard et al 2007a). *S. salmonis* from German rainbow trout was 75.1% similar to *S. vortens* from angelfish, and only 65.95 and 65.45% similar to *S. barkhanus* and *S. salmonicida*, respectively.

5.1.6 Result: Phylogenetic analyses

The alignment of the ssu rDNA from one isolate of *Spironucleus salmonis* (DQ394703) and closely related diplomonads consisted of 1235 characters when positions with gaps were removed. The resulting alignment was subjected to phylogenetic analyses using ML, ME and MP. All tree-building methods produced the same topology (see Fig. 6 in Saghari Fard et al. 2007a). *S. salmonis* was recovered as a sister taxon to *S. vortens* with strong bootstrap support. *S. salmonicida* and *S. barkhanus* appeared as the most basal taxa of the Hexamitinae sequences included.

5.1.7 Deposition of materials

A SEM stub (181-1) and a TEM block (2730-1) have been deposited at the Norwegian School of Veterinary Science (PO Box 8146 Dep., 0033 Oslo, Norway). The sequence of *Spironucleus salmonis* has been submitted to Genbank under Accession Nos. DQ394703 (Isolate 1) and DQ394704 (Isolate 2).

5.1.8 Discussion: Overview

Ultrastructural examination of diplomonad flagellates from the intestine of rainbow trout from Germany showed them to be *Spironucleus salmonis* (as described by Poynton et al. 2004), confirming the presence of this parasite for the first time in Germany. This study provided comprehensive characterisation of *S. salmonis*, including new details of surface ultrastructure, particularly recognition of the caudal projection. I also revealed new aspects of functional morphology of the cell. The ssu rRNA gene sequence from *S. salmonis* is clearly different from those of other piscine *Spironucleus* spp.

5.1.9 Discussion: Ultrastructural diagnosis

The ultrastructure of the diplomonad from the German rainbow trout was consistent with that of *Spironucleus salmonis* from Irish rainbow trout as described by Poynton et al. (2004), with one exception. In the present study, although the cytoplasm at the posterior end of the

cell was packed with free ribosomes, I did not see the 8-shaped sac of endoplasmic reticulum enclosing the ribosomes seen in some sections of *S. salmonis* from rainbow trout from Ireland (Poynton et al. 2004). This difference could be due to comparison of sections cut at different distances from the posterior end of the cell. Close to the posterior end, the endoplasmic sac was not present in *S. salmonis* from rainbow trout from Ireland (Fig. 5c in Poynton et al. 2004).

Previously, only internal ultrastructure has been used to distinguish *Spironucleus salmonis* from the other 4 well-characterised species of piscine diplomonads (Poynton et al. 2004). I now demonstrate that surface morphology can be used to distinguish species of piscine diplomonads. The unadorned surface of *S. salmonis* is distinct from that of *S. vortens*, which has a surface adorned with counter-crossing lateral ridges bearing tufts of microfibrils (Poynton et al. 1995). A caudal projection is borne by both *S. salmonis* and *S. torosa*; however, in *S. salmonis* there is a simple tapering posterior end, whereas in *S. torosa* the posterior end bears 2 raised ring-shaped structures (tori) (Poynton and Morrison 1990); *S. barkhanus*, and *S. salmonicida* do not bear a caudal projection, but 2 crescent-shaped structures (barkhans) (Sterud et al. 1997, Jørgensen and Sterud 2004, 2006).

5.1.10 Discussion: Phylogenetic analyses

The sequence of the *Spironucleus salmonis* ssu rRNA gene is given for the first time. The ssu rDNA sequence from *S. salmonis* could be clearly distinguished from all other sequenced *Spironucleus* spp. from fish. Sequencing the ssu rRNA gene therefore holds promise as a rapid method for identification of *Spironucleus* species from fish. The genetic differences observed between the isolates and the clones from the 2 German rainbow trout sampled in this study were probably due to amplification of different copies of the ssu rRNA gene, as observed by Keeling and Doolittle (1997) and Jørgensen and Sterud (2004). These differences may also be due to the lack of proof-reading of the *Taq* polymerase (Cline et al. 1996).

The phylogenetic analyses recovered *Spironucleus salmonis* as the closest relative to *S. vortens*. Based on the morphology of these 2 taxa, this was somewhat surprising. *S. vortens* has a

rather complex adorned surface (Poynton and Morrison 1990, Sterud and Poynton 2002), while *S. salmonis* is completely unadorned: this unadorned surface is more similar to that of *S. barkhanus* and *S. salmonicida*. However, the paraphyly of *Spironucleus* suggests that *S. barkhanus* and *S. salmonicida* are only distantly related to *S. salmonis*. This may be due to the increased rate of evolution observed for the diplomonads (Stiller and Hall 1999).

The basal position of *Spironucleus barkhanus* and *S. salmonicida* indicates that the unadorned surface probably is an ancestral state in *Spironucleus*, while the adorned surface of *S. vortens* is a derived character. The paraphyly of *Spironucleus* is consistent with descriptions in previous studies (Keeling and Doolittle 1997, Kolisko et al. 2005).

Discussing the ultrastructural similarities between *Spironucleus salmonis* and *S. barkhanus*, Poynton et al. (2004) kept the option open that these species could subsequently be synonymised. The present results show that their decision to retain them as separate species was correct.

5.1.11 Discussion: Functional morphology

Discharge of vacuoles (presumably containing waste digesta), at the surface of the body of *Spironucleus salmonis* has now been confirmed by SEM. My present and previous study (Poynton et al. 2004) indicates that the vacuoles can be discharged from regions with the heterogenous cytoplasm. Discharged vacuoles are not visible at the apex of the cell (where the cytoplasm is homogenous), nor at the extreme posterior of the cell (where there are densely packed ribosomes), nor along the flagellar pockets. Although there are few detailed studies on feeding and digestion in *Spironucleus* species, it is known that these flagellates are phagotrophic, and endocytosis is reported to take place at the top of the flagellar pocket (Kulda and Nohýnková 1978). However, the excretion of digestion products does not appear to have been documented previously. Discharging of digestive vacuoles has not been reported in other species of piscine diplomonads, suggesting different mechanisms of voiding the products of digestion. The cell surface of *S. salmonis* was confirmed as a simple plasma membrane (a Type I cell surface, according to the new classification of Becker (2000)).

Examination of electron micrographs of piscine diplomonads shows that each has a distinct caudal morphology, with separated emerging recurrent flagella. *Spironucleus barkhanus* bears barkhans, which deflect the emerging flagella away from each other (Sterud et al. 1997), *S. torosa* bears a long caudal projection with flagella emerging some distance from the tip (Poynton and Morrison 1990, Sterud 1998a,c), and *S. vortens* has broad counter-crossing lateral ridges with flagella emerging posterior-laterally (Poynton et al. 1995, Sterud and Poynton 2002). Consideration of non-piscine *Spironucleus* species also shows separated emerging recurrent flagella. For example in *S. meleagridis* and *S. muris* (infecting game birds and mice, respectively), the flagella emerge posterior-laterally, some distance from the posterior end of the cell, spiralling away from each other (Cooper et al. 2004 and Branke et al. 1996, respectively). Similarly, piscine diplomonads have distinct surface morphology, in *S. barkhanus*, *S. salmonis* and *S. torosa* this is simple, whereas in *S. vortens* this is complex.

I suggest that the lobes, of the entwined *Spironucleus salmonis* nuclei (Fig. 5.1.2b,c,e), are formed in response to the rigorous action of the closely apposed anterior-medial kinetosomes. The distally directed beats of the anterior flagella will be associated with movement of the kinetosomes (the bases of which are secured in reinforced depressions in the nuclear membrane, i.e. the kinetosomal pockets), and this may result in compensatory hydrostatic pressure within the nucleoplasm resulting in lobe formation. Lobes were present both adjacent to the kinetosomal pockets, and where the nuclei were apposed to each other. The entwined lobes should confer mechanical strength and integrity to the 2 nuclei and, in turn, provide stability for the anterior kinetosomes. Such mechanical strength and integrity would be advantageous, given that the apex of the *Spironucleus* cell is exposed to considerable internal mechanical forces, generated by the distally directed beats of the anterior flagella arising from the anterior-medial kinetosomes. I suggest that the deformability of the lobes depends upon the phase of the flagellar beat, and the rigour of the beat, which will be affected by such factors as the viscosity of the liquid through which the diplomonads swim.

Lobed nuclei are present in other piscine *Spironucleus* species (*S. barkhanus*, Sterud et al. 1997, 2003; *S. vortens*, Poynton et al. 1995, Sterud and Poynton 2002; *S. torosa*, Poynton and

Morrison 1990, Sterud 1998a; and in *Hexamita nobilis*, Li 1995, a possible *Spironucleus* species), although previous investigators have not commented upon their possible functional morphology. The nuclei of *Hexamita* species (Brugerolle 1974) and *Octomitus* species (Brugerolle et al. 1974) are not lobed and intertwined, but are simply appressed medially. Both these genera have anterior-lateral kinetosomes; thus, movement of the 2 clusters of kinetosomes will not produce intertwined lobes in overlapping nuclei. Furthermore, the apex of *Hexamita* and *Octomitus* cells may be subject to less internal mechanical stress than is the case in *Spironucleus*, which has anterior-medial kinetosomes.

I observed aggregates of glycogen extending longitudinally between the flagellar pocket and the striated lamina—an unusual location. The aggregated glycogen may have been transported to this location with other cytoplasmic components when the flagella pocket and the striated lamella were forming.

The present study of the dense bodies in the cytoplasm of *Spironucleus salmonis*, particularly their apparent ‘budding’ (Fig. 5.1.4b), is consistent with the secretory function previously suggested by Poynton et al. (2004).

5.1.12 Discussion: Host and geographical record

There are numerous reports of diplomonads in fish in Germany, including one of the earliest reports of infection in trout (Schmidt 1919), and an extended study of ecology and host specificity of a diplomonad originally called *Hexamita salmonis* (Sanzin 1965), and now more correctly called *Spironucleus salmonis*. Widely used fish pathology textbooks in Germany refer to *Hexamita* infections in salmonids (Roberts and Schlotfeldt 1985, Schäperclaus et al. 1990, Schlotfeldt 1991). However, comprehensive ultrastructural and molecular approaches for accurate identification of the parasites do not appear to have been used previously, and therefore the true identity of the parasites in these prior publications remains unknown.

The present study has demonstrated the presence of *Spironucleus salmonis* in a fish farm near Berlin. However, I do not imply that *S. salmonis* is a parasite newly introduced to Germany,

but rather that we have now—with modern techniques—assigned the correct name to the flagellate at this particular farm, which would probably have otherwise be referred to as *Hexamita salmonis* or *Octomitus truttae*. However, I emphasis that the name *S. salmonis* should only be used when a proper identification has been made using the comprehensive techniques described in the present paper. In the absence of ultrastructural and/or molecular characterisations, parasites should be recorded simply as diplomonads, since even genus cannot be adequately determined by light microscopy (Poynton and Sterud 2002).

5.2 Intestinal pH and the microhabitat preference of *S. salmonis*

5.2.1 Result: Overview

Both occurrence and density of *Spironucleus salmonis* differed significantly along the length of the intestine, being highest in the pyloric region and then decreasing posteriorly. The pH profile in uninfected and infected fish was characterized by more neutral conditions in the pyloric and posterior regions, and significantly more alkaline conditions in the anterior and middle regions of the intestine. In the pyloric region, the pH was not significantly different in uninfected and lightly, moderately and heavily infected fish. Among the 698 fish examined, 168 were infected (prevalence of infection 24%). There was no significant difference in size of infected and uninfected fish. Furthermore, there was no significant correlation between size of fish and pH in any of the 4 intestinal regions.

5.2.2 Result: Occurrence of infection and region

The occurrence of *Spironucleus salmonis* was significantly higher in the pyloric region (24% of fish were infected in this site) than in each of the other 3 regions (anterior 15%, middle 13% and posterior 10%) (χ^2 , $p < 0.001$), and the occurrence in the anterior region was also significantly higher than in the posterior region (χ^2 , $p < 0.005$) (Fig. 5.2.1).

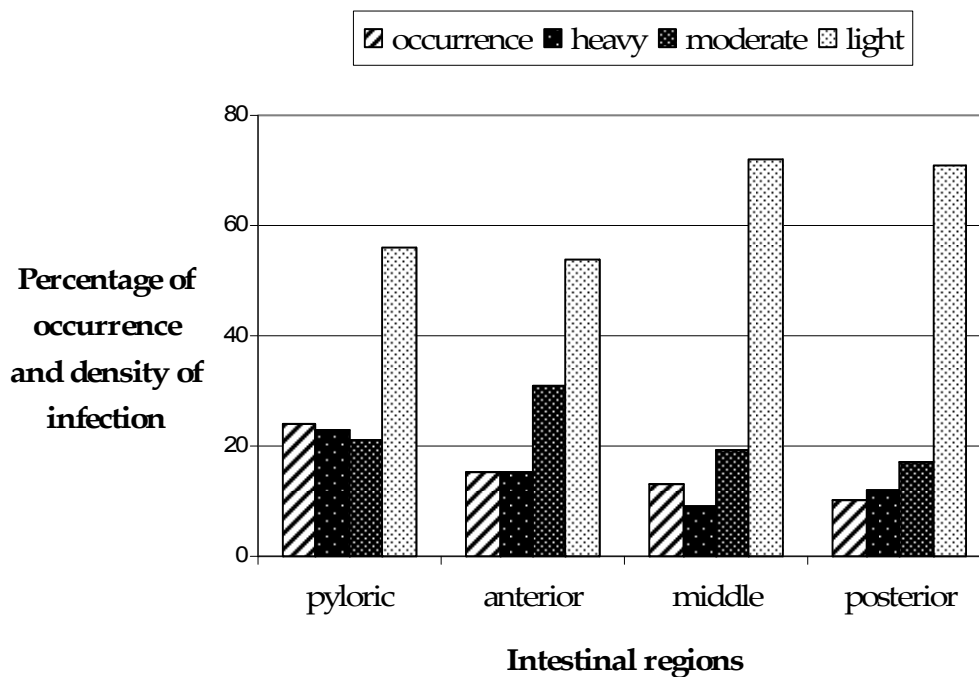


Fig. 5.2.1. *Spironucleus salmonis* infecting *Oncorhynchus mykiss*. Occurrence and density of the diplomonads in each of 4 regions of the intestinal tract of juvenile rainbow trout. Occurrence expressed as percentage of infected fish per region (n = 698 fish for each region). Densities expressed as percentage of infected fish with each density per region (n = 168 fish for pyloric region, n = 105 fish for anterior region, n = 89 fish for middle region, and n = 69 fish for posterior region). Reproduced with permission of Diseases of Aquatic Organisms.

5.2.3 Result: Density of infection and region

The density of *Spironucleus salmonis* in the pyloric region was significantly higher than in the anterior, middle and posterior regions (χ^2 , $p < 0.0001$), and density in the anterior region of the intestine was also significantly higher than in the middle and posterior regions (χ^2 , $p < 0.05$, $p < 0.005$ respectively). Results for 3 categories of density (upon which the χ^2 tests were based) per intestinal region are shown in Fig. 5.2.1.

In 38 fish heavily infected in the pyloric region, the density of infection in each of the 3 downstream intestinal regions was significantly higher than that in the 94 fish lightly

infected in the pyloric region (Mann-Whitney test, $p < 0.0001$). Furthermore, in 100% of fish heavily infected in the pyloric region, all 3 downstream regions were infected, whereas there were no fish infected in all 3 downstream regions when the pyloric region was lightly infected.

5.2.4 Result: pH and region in uninfected fish

Along the length of the intestine of 37 uninfected fish, pH ranged from 6.7 to 8.2 ($n = 37$) (Table 5.2.1). The pH was significantly lower in the pyloric and posterior regions than in the anterior and middle regions (ANOVA, $F(3,144) = 15.97$, $p < 0.0001$) (Table 5.2.1) (Fig. 5.2.2). At the individual level, among 37 uninfected fish, 79% adhered to this pH profile (defined as more neutral, more alkaline, more alkaline, more neutral, for pyloric, anterior, middle, and posterior regions, respectively).

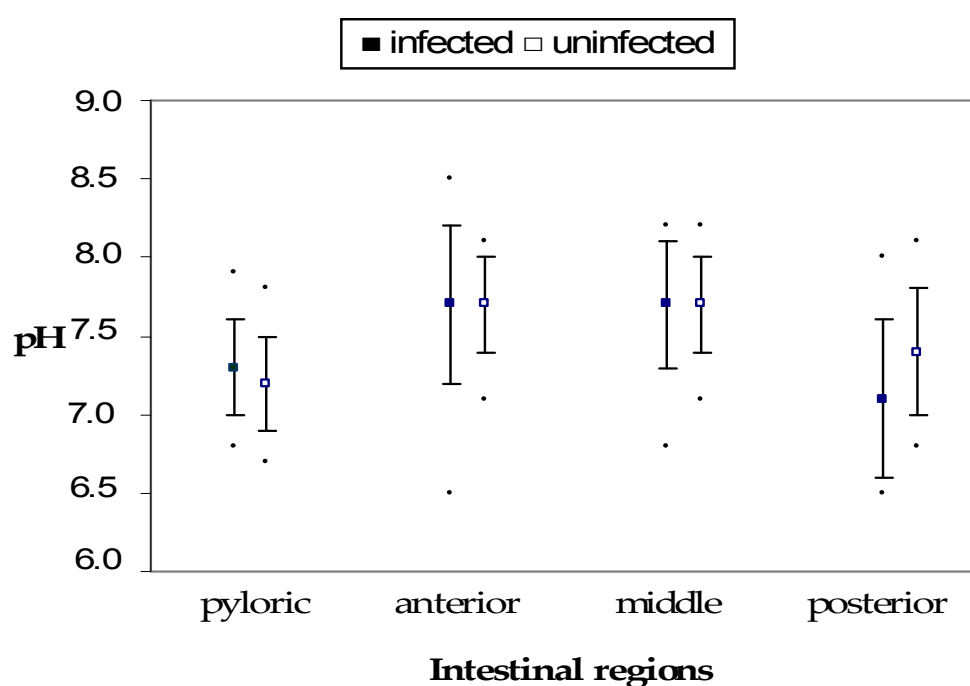


Fig. 5.2.2. *Oncorhynchus mykiss*. Mean pH \pm SD in each of 4 regions of the intestinal tract of 37 uninfected, and 21 infected rainbow trout with infection in all 4 regions of the intestine. d: pH range. Reproduced with permission of Diseases of Aquatic Organisms.

Table 5.2.1. *Oncorhynchus mykiss*. pH in 4 different intestinal regions of uninfected and *Spironucleus salmonis*-infected juvenile rainbow trout. –: mean and SD not available, as n = 1. Reproduced with permission of Diseases of Aquatic Organisms.

Intestinal regions	Uninfected fish ^a	Infected fish ^b	Infected fish ^c (density)		
			Light	Moderate	Heavy
Pyloric					
Range	6.7 – 7.8	6.8 – 7.9	6.8 – 8.0	6.8 – 7.9	6.5 – 8.0
Mean ± SD	7.2 ± 0.3	7.3 ± 0.3	7.4 ± 0.4	7.3 ± 0.3	7.3 ± 0.5
No. of fish	37	21	22	22	21
Anterior					
Range	7.1 – 8.1	6.5 – 8.5	7.0 – 8.6	6.5 – 8.5	6.7 – 8.0
Mean ± SD	7.7 ± 0.3	7.7 ± 0.5	7.7 ± 0.4	7.7 ± 0.5	7.4 ± 0.5
No. of fish	37	21	23	18	7
Middle					
Range	7.1 – 8.2	6.8 – 8.2	6.7 – 8.5	6.8 – 8.2	8.0 – 8.1
Mean ± SD	7.7 ± 0.3	7.7 ± 0.4	7.7 ± 0.4	7.7 ± 0.4	8.1 ± 0.0
No. of fish	37	21	33	8	2
Posterior					
Range	6.8 – 8.1	6.5 – 8.0	6.8 – 8.2	6.5 – 8.0	7.0
Mean ± SD	7.4 ± 0.4	7.1 ± 0.5	7.3 ± 0.4	7.1 ± 0.5	–
No. of fish	37	21	20	6	1

^a Fish which were uninfected in all 4 regions

^b Fish which were infected in all 4 regions

^c Fish which were infected in only 1, 2 or 3 regions; total numbers were 65 (pyloric region), 48 (anterior), 43 (middle) and 27 (posterior)

5.2.5 Result: pH and region in infected fish

Along the length of the intestine of 21 fish that were infected in all regions of the intestinal tract, pH ranged from 6.5 to 8.5 (Table 5.2.1). The pH was significantly lower in the pyloric and posterior regions than in the anterior and middle regions (ANOVA, $F(3,80) = 3.711$, $p < 0.05$) (Table 5.2.1) (Fig. 5.2.2). At the individual level, among 21 infected fish, 90% adhered to this pH profile (more neutral, more alkaline, more alkaline, more neutral).

5.2.6 Result: pH in uninfected and infected fish per region

There were no significant differences in pH between 37 uninfected and 21 infected fish in the pyloric, anterior and middle regions; however, in the posterior region, the pH was significantly more alkaline in uninfected fish than in infected fish (t -test, $t(56) = -2.58$, $p < 0.05$) (Table 5.2.1).

5.2.7 Result: pH and density of infection in different regions

In the pyloric region, there were no significant differences in pH between the 37 uninfected fish, 22 lightly, 22 moderately, and 21 heavily infected fish (Table 5.2.1). The low number of fish with moderate and heavy infections in anterior, middle and posterior regions precluded statistical analysis (Table 5.2.1).

5.2.8 Result: Size of fish and infection

Among the 698 fish, there was no significant difference in total length or weight between 530 uninfected fish and 168 infected fish.

5.2.9 Result: Size of fish and pH

In 37 uninfected fish, there were no significant correlations between size of the fish (total length or weight) and pH, in any of the 4 intestinal regions.

5.2.10 Discussion: Microhabitat preference

Spironucleus salmonis, though found throughout the intestinal tract of juvenile rainbow trout, was concentrated in the pyloric region, with both occurrence and density significantly decreasing posteriorly. This distribution was consistent with previous studies by Davis (1926), Ferguson (1979), and Moore (1922a,b), and contrasted in part with the study by Uldal and Buchmann (1996), which did not report significant differences in occurrence and density between the pyloric and anterior regions. These differences may be due to the sizes of fish,

viz. juveniles in our study (mean length 12.7 cm) and fry in the study by Uldal and Buchmann (1996) (mean length 4.1 cm).

The microhabitat preference in *Spironucleus salmonis* can initially help diagnosis of the flagellate, since the 4 other well characterized *Spironucleus* species have other site preferences (see chapter 2, Table 2.1).

5.2.11 Discussion: pH profile in uninfected fish

The pH in the intestinal tract of uninfected trout in the present study, 6.7 to 8.2, partly overlapped the range of 7.0 to 9.0 previously reported for salmonids (Steffens 1989). Along the intestinal tract, we observed a distinct variation in pH (hereafter referred to as the pH profile), with means of 7.2, 7.7, 7.7 and 7.4 in the pyloric, anterior, middle and posterior regions, respectively. Longitudinal pH profiles have been reported for other fish with stomachs, including other salmonids, channel catfish *Ictalurus punctatus*, Mozambique tilapia *Oreochromis mossambicus*, and sea bream *Sparus aurata* (Page et al. 1976, Maier and Tullis 1984).

The longitudinal pH profile in the juvenile rainbow trout reflects the diverse digestive processes and dietary physiology in the different regions. In the pyloric region, the acidic chyme from the stomach is mixed with bile and pancreatic secretions including zymogens and bicarbonate buffering compounds, and the pH becomes more neutral. The anterior and middle intestines are part of the descending intestine, having similar anatomy, physiology, and pH. Here conditions become more alkaline as buffering decreases. In the posterior intestine, conditions become more neutral.

The wide variations in pH within regions of the intestinal tract of the rainbow trout (Table 5.2.1) might be explained by differences in the time interval between feeding and examination, since pH varies as a function of feeding time (Maier and Tullis 1984, Deguara et al. 2003).

5.2.12 Discussion: Possible factors affecting microhabitat preference

The range of pH along the intestinal tract of uninfected fish, 6.7 to 8.2, lay well within the rather broad pH tolerance of 5.5 to 9.0 previously reported for putative *Spironucleus salmonis* in culture (Buchmann and Uldal 1996). Thus, it is not surprising that the trophozoites were present in all 4 regions of the intestinal tract. I also note that the pH conditions in infected fish, 6.5 to 8.5, were close to the pH conditions of 6.5 to 7.0 and 7.5 to 8.0 (the latter being most suitable) that supported population growth of putative *S. salmonis* in culture (Buchmann and Uldal 1996). However, the pH in the preferred pyloric region (mean \pm SD: 7.2 ± 0.3 in uninfected fish, 7.3 ± 0.3 in infected fish) was lower than the reported optimum for putative *S. salmonis* in culture (7.5 to 8.0) (see discussion below on optimum pH for *S. salmonis*).

The highest numbers of *Spironucleus salmonis* flagellates were seen in the near neutral conditions of the pyloric region (mean pH 7.3), and the lowest numbers were seen in the near neutral conditions of the posterior region (mean pH 7.1). This distribution might be explained by the more alkaline pH of the anterior and middle regions of the intestine (mean pH 7.7 for both), being less favourable for the diplomonads, reducing occurrence and density in these regions, and allowing few diplomonads to pass to the posterior region. However, data from an *in vitro* study of putative *S. salmonis* (Buchmann and Uldal 1996) showed that pH conditions of 7.5 to 8.0 were optimum for population increase. Thus it is most unlikely that the pH of 7.7 in the anterior and middle regions were the reason for the reduced numbers of *S. salmonis*.

I am unable to confirm from the *in vivo* studies whether the preference of *Spironucleus salmonis* for the pyloric region is related to pH. As noted above, I observed a pH range of 6.8 to 7.9 (mean 7.3) in infected fish, 6.7 to 7.8 (mean 7.2) in uninfected fish, and previous *in vitro* studies of putative *S. salmonis* give 7.5 to 8.0 as the optimum for population growth (Buchmann and Uldal 1996) (see discussion below on optimum pH for *S. salmonis*). It is indeed very likely that other factors, such as bile, bacterial fauna, and nutrient content of the digesta play a key role in determining the microhabitat preference of *S. salmonis*. These other factors may be dominant, or may act in concert with pH.

Bile enters the pyloric region via the bile duct that passes among the pyloric caecae (Willers 1991), and its concentration is expected to decrease posteriorly. *In vitro* studies show that bile in low concentrations (30 to 960 mg l⁻¹) slightly enhanced propagation of putative *Spironucleus salmonis* (Buchmann and Uldal 1996); however, statistical tests were not performed, so I do not know if the enhanced propagation was statistically significant. Thus, it may be expected that, *in vivo*, bile in the pyloric region also enhances propagation, possibly mediated by the gallic acids that emulsify fat (Steffens 1989), and/or the bile salts (Denton et al. 1974).

A further anterior posterior gradient occurs in the bacterial fauna. There is a progressive decline in numbers of aerobic bacteria posteriorly, and anaerobes are generally restricted to the upper intestine (Austin and Al- Zahrani 1988). I suggest that the bacterial milieu of the pyloric region may be more favourable for *Spironucleus salmonis* than that of more posterior regions. Although the way in which bacteria may affect the trophozoites is not known, we suggest the mechanism is indirect (perhaps via microbial digestion), since bacteria have not been reported to attach to or lie within the cytoplasm of *S. salmonis* (Poynton et al. 2004).

The nutrient content of the digesta decreases posteriorly, a further factor correlated with distribution of *Spironucleus salmonis*. In the most posterior parts of the intestinal tract, digestive and absorptive functions are diminished (Smith 1989); thus, there may not be adequate nutrients for the diplomonads.

In *Spironucleus salmonis*, I have shown that while the presence of infection reflects tolerable pH, density of infection is not closely related to pH, since there was no correlation between density and pH along the length of the intestine, and thus a causal relationship is unlikely.

5.2.13 Discussion: Optimum pH for *Spironucleus salmonis*

The present *in vivo* study indicates that the optimum pH for *Spironucleus salmonis* may be 7.3, since this was the mean pH in the pyloric region where the diplomonads were concentrated. My determination of pH was accurate to within 0.2. Therefore, I can state that, while our *in*

vivo data suggests an optimum pH for *S. salmonis* of 7.3, the optimum may lie within the range of 7.1 to 7.5.

This *in vivo* result contrasts with the results from *in vitro* studies for putative *Spironucleus salmonis* by Buchmann & Uldal (1996), who reported a pH optimum of 7.5 to 8.0, based on highest population density. If I assume that the same species of diplomonad was investigated in this *in vivo* study and in the previous *in vitro* study (Buchmann and Uldal 1996), how might these apparently different optima be explained? In principle, it is likely that the pH recorded *in vivo* is closer to the optimum than that determined from culture, since *in vitro* conditions represent a compromised environment for trophozoites. It is also possible that the parasites we studied from rainbow trout in Germany are a different species or strain from that studied by Buchmann and Uldal (1996) from rainbow trout in Denmark. Do different sub-species or strains, if they exist, have different pH optima?

The suitability of pH 7.3 for *Spironucleus salmonis* has not previously been determined. The results of an *in vitro* investigation of putative *S. salmonis* by Buchmann and Uldal (1996) appeared to exclude pH conditions between 7.0 and 7.5. Moreover, it is not known whether pH 7.5 or 8.0 was more favourable for growth, since pooled data was presented. Additional support for a true optimum pH being lower than 7.5 to 8.0 comes from our colleague's *in vitro* studies, where cultures at pH 7.0 to 7.5 were more successful than those at pH 7.5 to 8.0 (Cheng 2006). However, caution should be exercised when comparing results of our *in vitro* cultures with those of Buchmann and Uldal (1996), since as mentioned, they may have contained different subspecies or strains of *S. salmonis*, and furthermore, the culture conditions were not identical. Since this *in vitro* study was targeted at yielding stock cultures for immunology experiments, I did not determine optimum pH.

5.2.14 Discussion: Size of fish and infection

My finding that there were no significant differences in total length or weight of uninfected and infected juvenile rainbow trout (mean total length 12.7 cm) contrasts with the significant

weight reduction reported for infected rainbow trout fry (mean total length 5.5 cm) by Uldal and Buchmann (1996).

As discussed earlier, I have also noted that in the juvenile fish, occurrence and density were significantly higher in the pyloric region than elsewhere, whereas in fry there were no significant differences between pyloric and anterior regions (Uldal and Buchmann 1996). These differences demonstrate that the nature of *Oncorhynchus mykiss* – *Spironucleus salmonis* relationship is dependant upon the size of the fish. We suggest that key factors are development of immunity (affecting the consequences of the infection, with acquired immunity developing in the juvenile fish and allowing them to cope more successfully with the infection), and development of the gut (affecting the distribution of the flagellates).

5.2.15 Recommendations

A number of questions about the factors determining microhabitat preference of *Spironucleus salmonis* remain unresolved, and I now offer recommendations for further studies to refine and extend the approach that I presented. Refinements should include (1) standardizing the intervals between collection from the farm and examination, and between feeding and examination, thus minimizing effects of stress and changes in pH respectively; (2) recording density quantitatively (actual numbers of flagellates), by slowing or stopping movement of the active flagellates, via photography or adding formalin to the preparation; and (3) using microelectrodes to measure luminal pH (Brune and Kühl 1996), and placing them precisely in the posterior region in order to measure pH in the rectum only (posterior to the annulo-spiral septa). Since age can be expected to have a marked impact on development of the gut, and hence the environment for the diplomonads, I also recommend that future studies should extend the present work on juvenile rainbow trout by comparing pH and microhabitat preference of *S. salmonis* in both fingerlings and sub-adults.

Extension of the present approach should include (4) investigating the pH preference of *Spironucleus salmonis* by exposing organisms in culture to pH gradients, and documenting their speed and direction of movement, and (5) ascertaining the importance of bile for

growth of *S. salmonis*, which can be addressed by measurement of bile concentration *in vivo*, and additional *in vitro* studies with statistical treatment of data. Successful determination of optimum pH and bile requirements will enhance the efficiency of routine *in vitro* culture of *S. salmonis*.

In a broad sense, knowledge of *Spironucleus salmonis* microhabitat preference and determining factors may play a key role in predicting the likely impact of changes in diet on density of diplomonads. In recent years, there have been increasing efforts made to develop diets for carnivorous fish, such as trout, that have increasing amounts of plant protein, since fish meal production is stagnant or decreasing (Cheng et al. 2003). It may be expected that such changes in diet will bring about changes in the intestinal milieu within which the diplomonads live. Improved understanding of the environmental requirements of *S. salmonis* will help predict whether changes in diet may result in increased densities of the diplomonads, which may be pathogenic in young fish.

5.3 Multi-functionality of flagella in *Spironucleus salmonis*

5.3.1 Result: Role of media

In all 7 cultures using the newborn calf serum, we observed numerous cysts, most of which were clustered, and relatively few trophozoites. Whereas, in the 20 cultures using the bovine serum, we observed numerous trophozoites; clusters of cysts were never observed, and solitary cysts were not recognised.

5.3.2 Result: Attachment of posterior flagella to each other and to debris

Normal trophozoites swam actively, with their 6 anterior flagella actively beating, and the 2 posterior flagella trailing behind the body (Fig. 5.3.1a). The presentation of an adhesive flagellar surface was initially recognised by the attachment of the tip of the posterior flagella to other trophozoites and / or to debris (Fig. 5.3.1b,c).

Attachment of adjacent swimming trophozoites to each other was via alignment of the posterior flagella (Fig. 5.3.1d). Sometimes the adhesion was weak enough to allow the trophozoites to attach to each other, and then detach. In other cases adhesion was strong enough to allow two trophozoites attached only by the tips of their posterior flagella, to swim together, with motility generated by the anterior flagella, sometimes with one trophozoite leading and the other following, and then switching. Adhesion appeared to present first at the distal tip of the posterior flagella, and then extend proximally towards the body.

Trophozoites attached to debris from the digesta of the initial inoculum, by the tip of one or both posterior flagella. If the debris was small, the trophozoites continued to swim, pulling the debris behind them. However, attachment to large pieces of debris inhibited swimming, and the tethered trophozoite whirled on the spot (Fig. 5.3.1c). At low magnification, the tethered whirling of many trophozoites attached to large pieces of debris was recognized as a flickering halo around the debris.

5.3.3 Result: Clustering of trophozoites prior to encystment

As the trophozoites aggregated by their posterior flagella, clusters were formed, their appearance reminiscent of a cluster of balloons (Figs. 5.3.1d - f). Some trophozoites are no longer pyriform, but more spherical as they prepare to encyst (Fig. 5.3.1f). In clusters in which the digesta formed the nidus / foundation, some trophozoites were attached directly to the digesta (Fig. 5.3.1g), and others were attached to other trophozoites, resulting in clusters larger than the piece of digesta (Fig. 5.3.1h). Sometimes large clusters of cells were formed, comprising approximately 45-50 individuals (Fig. 5.3.1h). Typically the founder cells at the center of the cluster began to round up and encyst, while new pyriform cells joined the cluster at the periphery (Fig. 5.3.1f,h).

5.3.4 Result: Timing of adhesion and clustering

Adhesion and clustering could occur within as little as 30 minutes after inoculation of the primary culture, as was the case in a single newborn calf serum culture, which was inadvertently subject to heat stress. In the remaining newborn calf serum cultures, adhesion and clustering was first observed at day 2 post inoculation in 5 cultures, and at day 10 in one culture. In all 7 cultures, at the time point when adhesion and clustering were first observed, a mixture of cell morphologies were present, free swimming trophozoites, trophozoites with sticky tips to the posterior flagella, small clusters, large clusters, and both clustered and solitary cysts; most cells were in clusters. Within 24 hours of adhesion and clustering first being observed, all cells had encysted, most of which were clustered, the minority were solitary.

5.3.5 Result: Encystment

During encystment, the clustered trophozoites changed from pyriform to spherical (Fig. 5.3.1f-h). The late phase of encystment was recognised in light microscopy preparations by a refractile cyst wall forming around the cell (Fig. 5.3.1i). Occasionally small blebs were seen on the surface of the cell, tentatively identified as encystation vesicles (Fig. 5.3.1i).

5.3.6 Result: Fine structure of the surface of clustered trophozoites and cysts

In recently attached pyriform trophozoites, the unadorned surface of the cell was generally smooth, and bore small raised areas (Fig. 5.3.2). The posterior flagella, and the portions of the anterior flagella that extended posteriorly, were all tightly aligned (Fig. 5.3.2). Cells at the periphery of large clusters were oriented with their anterior ends outwards (recognized by the emerging triads of anterior flagella), consistent with the cells being attached to each other, and /or to digesta, by their posterior ends (Fig. 5.3.2).



Fig. 5.3.1. Attachment, clustering and late encystment in *Spironucleus salmonis* in *in vitro* culture. (a) Normal motile trophozoites, pre-adhesion. (b) Motile trophozoite in early stage of adhesion, note that the tips of the posterior flagella (arrows) attached to each other. (c) Trophozoite tethered via the tip of the posterior flagella to a large piece of debris from digesta. (d) Two trophozoites attached together by most of the length of their posterior flagella. (e) A small cluster of three trophozoites, attached by their posterior flagella, and reminiscent of a bunch of balloons. (f) Cluster of 9 trophozoites; note that some are no longer

pyriform, but more spherical as they prepare to encyst. (g) Clusters of trophozoites attached to large pieces of digesta, note that some individuals are attached directly to the digesta, and other individuals are in turn attached to them. (h) Cluster of approximately 45 individuals, note that the recently attached trophozoite (in periphery of cluster) is pyriform and attached to the cluster by its posterior flagella, in contrast the older members of the cluster (in the middle of cluster) are spherical and flagella can not easily be recognized. (i) Attached trophozoite in the late phase of encystment, as evident by the blebs (arrows) and refractile cyst wall forming around one end of the cell. The two other trophozoites are still pyriform, and in attachment phase (prior to encystment). Light micrographs of glutaraldehyde-fixed material. Scale bars = 10 μm .

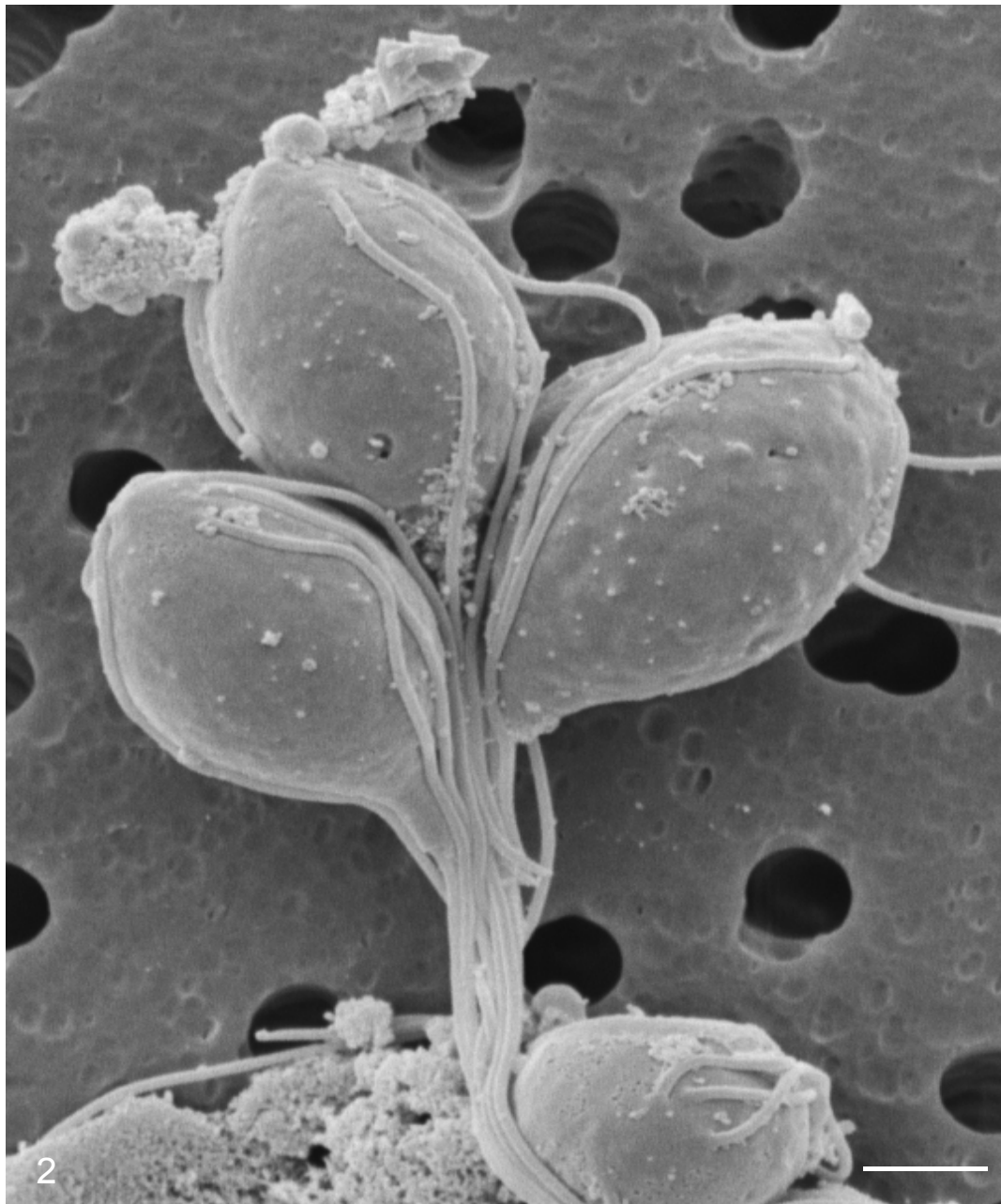


Fig. 5.3.2. Attachment of *Spironucleus salmonis* trophozoites in *in vitro* culture. Three attached pyriform trophozoites, note that the unadorned surface of the cell is generally smooth, and bears small raised areas; the posterior flagella, and the portions of the anterior flagella that lie posteriorly, are all tightly aligned. Scanning electron micrograph. Scale bar = 2 μm . Reproduced with permission of Dr. Erik Sterud, National Veterinary Institute, Oslo, Norway.

As the clusters matured, the cells became more spherical, oriented with their anterior ends outwards at the periphery; the anterior flagella lay along the body surface, over the body, and they do not appear to attach to each other (Figs. 5.3.3). In scanning electron microscopy,

the cysts wall appeared as a pale grey veil over the darker grey trophozoite (Fig. 5.3.3A). The cyst wall was not assembled synchronously over some cells, but as an ever enlarging field (comes down or round or over), which gradually covered the cell, including the anterior flagella lying upon the surface (Fig. 5.3.3 B,C,D).

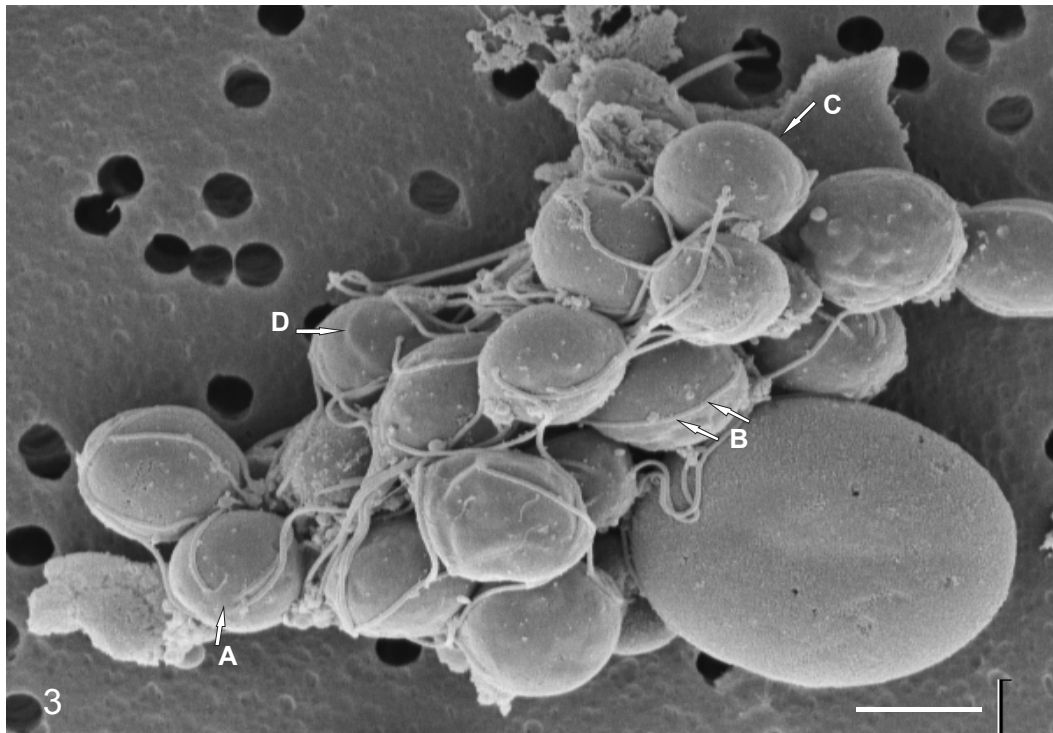


Fig. 5.3.3. Clustered and encysting *Spironucleus salmonis* in *in vitro* culture. A large cluster of approximately 20 cells, note the orientation pattern, with cells at the periphery oriented with their anterior ends outwards (recognized by the emerging triads of anterior flagella) (A), consistent with the cells being attached to each other, and /or to digesta, by their posterior ends. In this cluster, most of the cells have become spherical, and several are in the late phase of encystation, when they are partially (B) or completely (C) covered by the cyst wall. The cyst wall was not assembled synchronously over the cell, but as an ever enlarging field (comes down or round or over) gradually covered the cell. The anterior flagella lying upon the surface of the cell become covered by the cyst wall; the undulations of a flagellum can be clearly seen beneath the cyst wall in one cell; note that the cyst wall appeared as a pale grey veil when compared with the darker grey surface of the trophozoites (D). The large disc-like

structure is probably a blood cell. Scanning electron micrograph. Scale bar = 5 μm . Reproduced with permission of Dr. Erik Sterud, National Veterinary Institute, Oslo, Norway.

5.3.7 Discussion: Overview

The present study appears to be the first to document attachment by the posterior flagella in diplomonads, although flagellar attachment is known in kinetoplastids. The results show multi-functionality of the posterior flagella in *S. salmonis*, and their ability not only in motility, but also in adhesion. Cluster of trophozoites were formed by attaching posterior flagella, and probably later by cell membrane. The results, and that of the ultrastructural study (section 5.1), indicate that the plasma membrane of the cell may have five functional domains; (i) anterior flagellar membrane, (ii) posterior flagellar membrane, (iii) anterior pellicular membrane, (iv) middle and posterior pellicular membrane, and (v) flagellar pocket membrane.

The adhesion ability of flagella and cluster formation *in vivo* would provide an effective mechanism to enhance transmission through the water. Gas bubbles and plant debris associated with colonies would help to maintain buoyancy, and hence transmission. Clusters would ensure the simultaneous ingestion of numerous cysts, presumably exceeding the maximum effective dose for initiating a new infection. Although minimum infective oral dose has not been established for piscine diplomonads, the number of individual diplomonads in the clusters I observed (approximately 50) lies within the range for minimum infective dose for avian and mammalian diplomonads.

Since the effective treatment against trophozoites, metronidazole, is prohibited in many countries, targeting the cyst can be a solution to protecting fish against new infection. The possible suggestion is that the transmission of the life cycle can be disrupted by mechanical and/or chemical break up of the adhesive clusters. The anti adhesion therapy is well established for bacteria treatments.

5.3.8 Discussion: Multi-functionality of flagella – possible mechanisms

In the direct life cycle of *Spironucleus*, the motile trophozoites commonly encountered amidst the digesta in the intestinal lumen of the host, alternate with resistant cysts which transmit the infection through the external environment, with new infections acquired by oral ingestion. *In vivo* observations have reported both solitary cysts of *Spironucleus*, as in early descriptions of *S. salmonis* from trout (Davis 1926, Moore 1922a,b), and clusters of cysts in the intestinal mucus as is the case in *S. meleagridis* from ring necked pheasants, *Phasianus colchicus* (Wood and Smith 2005).

However, the possible role of flagella in facilitating clustering in encysting *Spironucleus* has apparently only been documented *in vitro* by Uldal (1996). The *Spironucleus* trophozoite has six anterior flagella, which are considered as locomotory, and two posterior flagella that are trailing flagella (Brugerolle and Lee 2002, Woo 2006). The present study demonstrates that adhesion appears only to be a property of the two posterior flagella.

This multi-functionality of flagella in *S. salmonis* is in contrast to the flagella of the well-studied diplomonad, *Giardia*, in which the ventral disc is used for attachment (Adam 2001). However, it is consistent with flagella in parasitic kinetoplastids (i.e. flagellum of the African trypanosome, *Trypanosoma brucei* known as an adhesion organelle to attach to host tissue (Vaughan and Gull 2003)). In the trypanoplasm-like flagellate *Jarrellia atramenti*, a commensal living in the blow hole of the pygmy sperm whale (*Kogia breviceps*), flagella attach to host material via the tip of the posterior flagellum (Poynton et al. 2001). The flagella in bi-flagellated green alga *Chlamydomonas*, has also adhesion function, which brings the mating type *plus* (*mt+*) and mating type *minus* (*mt-*) gametes to fusion of their plasma membranes, and forming a single cell with four flagella, the quadric-flagellated zygote (Misamore et al. 2003).

Molecular adhesions: Determining the molecular mechanisms of attachment and clustering in *S. salmonis* can begin by a search for factors known to be involved in flagella attachment of other organisms, especially the ones which are closely related phylogenetically to *Spironucleus*. In the ventral disc of *Giardia*, the protein “vinculin” has been identified that

binds α -tubulin and mediates attachment of actin filaments to membrane sites (Narcisi et al. 1994). In addition to ventral disc, on the outer surface of the excyzoite (intermediate stage between a trophozoite and a cyst), the protein “ α -1 giardin” has been distinguished, which is a calcium-binding protein with lectin activity (Weiland et al. 2003).

In trypanosomatid flagellate parasites, the attachment always takes place through the flagellum (Kohl and Gull 1998). The protein analysis of extracted cytoskeletons in *Trypanosoma congolense* suggested a major involvement of a 70k protein in the attachment function of flagella (Beattie and Gull 1997). In *Chlamydomonas*, adhesion and fusion of the plasma membranes of mating type plus and mating type minus gametes is accomplished via adhesion protein, agglutinins, on the surfaces of their flagella (Wang and Snell 2003).

Flagellar pocket: In my observation, only the posterior flagella were involved in the attachment of trophozoites to each other or debris. One can expect that in *Spironucleus*, the additional role of flagella in adhesion would only be played by the posterior flagella, since it is only these that pass through a flagellar pocket. In trypanosomatids, it is known that some secretory products received by, and inserted into the flagellar pocket, can migrate to the flagella, and some proteins released into the flagellar pocket remain in solution (De Souza 2006). It is known that the flagellar pocket of *Spironucleus* is also the site for endocytosis and exocytosis (Woo 2006); thus the flagellar pocket of *S. salmonis* may also play a key role in effecting adhesion.

Intra-flagellar transport (IFT): The beginning point of attachment process in the posterior flagella of *S. salmonis* trophozoites was the tip of the posterior flagella. It is of interest to know that recently has been discovered a phenomenon named intra-flagellar transport (IFT) in *Chlamydomonas*, which is a motility process of non-membrane-bound particles (IFT particles) ferried along flagellar microtubules from the base to the tip of the flagella (Cole 1999, Marszalek and Goldstein 2000, Rosenbaum et al. 1999). Since the posterior flagella in *S. salmonis* are located at flagellar pocket, the site for secretory and endocytic activities, it can be speculated that the adhesive molecules are synthesized in base of flagellar pocket, then are

transferred from the base to the tip of the posterior flagella; thus begins the process of attachment.

The 8-shaped sac at the posterior end of *S. salmonis*: In addition to the proposed key role of the flagellar pocket in effecting adhesion of the posterior flagella, it is also possible that an organelle, apparently unique to *S. salmonis*, is also involved. A sac of densely packed free ribosomes surrounds the posterior part of the flagellar pockets (Poynton et al. 2004), and this may be a site for synthesis of adhesive molecular which can be transported to the posterior flagella to effect adhesion.

The paraflagellar rod (PFR): In kinetoplastids, the specific organelle named the paraflagellar rod (PFR) has been considered to be involved in attachment (Bastin et al. 1996). The PFR is a complex and highly organized lattice-like structure that runs adjacent to the axoneme throughout the flagella length (Bastin et al. 1996). The attachment is usually mediated at the distal flagellar tip, where the anterior tip of the flagellum enlarges and contains the PFR and additional filaments that emerge from the main PFR. This suggests that the PFR could play a role in the process of tissue attachment (Maga and Le Bowitz 1999). Although, the PFR has not been reported in *Spironucleus* species, there is a recent report of dense electron plaques in the posterior flagella of *S. salmonis*, between the axoneme and the striated lamina, by Poynton et al. (2004). It could be postulated that they have a similar role of PFR in kinetoplastid flagellates, and contributes to facilitating and stabilizing flagella attachment to debris or other trophozoites.

5.3.9 Discussion: Functional domains of the *S. salmonis* plasma membrane

This hypothesis and explanation that in *Spironucleus*, the adhesion only occurs by the posterior flagella, which pass through a flagellar pocket, is based on the assumption that the flagellar pocket membrane in the diplomonad cell has similar functions to that in the trypanosomatid cell. In *Trypanosoma*, the plasma membrane has three different functional domains, the pellicular membrane, the flagellar membrane, and the flagellar pocket membrane (Bastin et al. 2000, Gull 2003). In the latter, the density of the glycoprotein coat is

different, and it is the only place of endocytosis and exocytosis, it is the major site where cell surface receptors are located, and it is the location for the mechanisms of targeting to different domains (Bastin et al. 2000, Gull 2003).

The present observations of the attachment of trophozoites prior to encystment in *S. salmonis*, coupled with the previous ultrastructural studies of the normal trophozoites (Poynton et al. 2004), allow us to now define five distinct functional domains of the plasma membrane of *S. salmonis*. The continuous plasma membrane appears to have the following five domains: (i) anterior flagellar membrane, which is non-adhesive; (ii) posterior flagellar membrane, which is potentially adhesive; (iii) anterior pellicular membrane, adjacent to the flagella and lying over homogenous cytoplasm, discharging vacuoles have not been observed in this area despite numerous SEM and TEM studies; (iv) middle and posterior pellicular membrane, lying over heterogenous organelle-rich cytoplasm, and through which vacuoles discharge; and (v) flagellar pocket membrane, previously reported as the site for endocytosis and exocytosis (Woo 2006), and now also possibly implicated in targeting adhesion between the different flagellar domains.

5.3.10 Discussion: Cluster formation

Clusters were sometimes very large, greatly exceeding the size expected if the trophozoites could only attach to each other by the tip of their posterior flagella (Figs. 5.3.1f-h). This suggests to us that: (i) once the posterior flagella become adhesive, they remain so, i.e. there is no feedback to limit adhesion to the first two trophozoites that come together; (ii) eventually the entire surface of posterior flagella becomes adhesive; (iii) the addition of trophozoites to the cluster may stimulate production / expression of adhesins, and (iv) swimming trophozoites may be actively attracted to join the forming clusters (changes in swimming behavior in response to a stimulus has already been documented in the piscine diplomonad *Spironucleus vortens* (Poynton et al. 1995), so could also be anticipated in *S. salmonis*).

5.3.11 Discussion: Encystment *in vitro* compare with *in vivo*?

It is intriguing to speculate whether attachment and clustering prior to encystment also occurs *in vivo* in *S. salmonis* or other *Spironucleus* species. The early descriptions of *S. salmonis* report solitary cysts being found in the intestine of fish (Davis 1926, Moore 1922a,b), but not cyst clusters. It is possible that clusters of cysts of *S. salmonis* have been existed in Davis (1926), and Moore (1922ab) studies, but have been disrupted during sample preparation, or had been hidden in the intestinal thick mucus. To the best of our knowledge, there are no reports of cluster formation in other *Spironucleus* species, except in *S. meleagridis* from ring-necked pheasants, which it has been noted that the cysts “often occurring in clusters” in the intestinal mucus (Wood and Smith 2005). However, it is not known whether the *S. meleagridis* clusters were simply cysts lying together because of the sticky nature of mucus, which covered them, or whether clusters had been formed due to an attachment process like that which we have now described.

It could be speculated that the attachment and clustering prior to encystment occurs based on a latent ability provoked by unusual conditions, such as exposure to some particular component(s) of the newborn calf serum encountered in culture, not an inevitable part of the life cycle. The different behavior of the trophozoites in the two culture media, with attachment proceeding encystment being commonly seen in the newborn calf serum media, but never in the fetal bovine media, was consistent with similar experiments by Uldal (1996), and Buchmann and Uldal (1996) respectively. However, the specific factors in the newborn calf serum that induce attachment prior to encystation are not known.

There was also a different between my observation and what Uldal (1996) reported; in my experiments, I first observed attachment and clustering prior to encystment as soon as 30 minutes after inoculation, and usually 2 – 10 days after inoculation. In contrast, Uldal (1996) reported that encystment, preceded by clustering, began 20 days after inoculation.

Increased morphological complexity in response to specific factors of environmental conditions has been reported for other flagellates. For *Giardia*, which is phylogenetically close to *Spironucleus*, cholesterol starvation, addition of bile salts and fatty acids, have all been

implicated in induction of encystation *in vitro*, though their relative importance is controversial (Adam 2001). For *Cryptobia salmositica*, a hemoflagellate, when Li and Woo (1991) investigated *in vitro* affects of fetal bovine serum (FBS) and glucose on multiplication of this pathogen, they were found rosette colonies formed only in media supplemented with high levels of FBS (20%-30%), whereas in the other media, no colonies were observed.

In my experiment, not only the Uldal (1996) media effected formation of the clusters; but also the different time for induction of clustering. The very rapid induction of adhesion and attachment (within 30 min after incubation) were seen only in one culture, probably due to a temperature stress. The contents of pyloric region of infected fish had been held at high temperatures in the lab (about 28.9 °C) for an unusually long period (30 min) of while other samples were prepared, and then they were inoculated into the culture medium at 10 °C. A 30 min exposure to temperatures approaching 30 °C would represent a considerable stress for *S. salmonis*, which is adapted to lower temperatures of 5 – 10 °C (Buchmann and Uldal 1996).

One can speculate that some specific conditions *in vivo* may happen to switch on trophozoites for attachment and clustering; when the fish dies, and the conditions in the intestine change, perhaps provides the chemical stimulus for switching on the attachment molecules in trophozoites, and occurring the attachment, clustering, and release – when the fish decomposes – of clusters of cysts into the environment, and passing to a new host by ingestion

5.3.12 Discussion: Cyst wall in *S. salmonis*

Brugerolle et al. (1980) described the cyst wall of *S. muris* (from mice) as “thin transparent cystic envelope”, using light microscopy, which is consistent with our observation for *S. salmonis* cyst in culture. The TEM observation of *G. lamblia* cysts has been shown that the cytoplasm contained abundant peripheral vesicles with round or tubular profile. In addition, the large vacuoles have also been found mostly at the periphery of the wall, were they expanded progressively in apposition to the plasma membrane, adopting the appearance of

flattened sac (Chávez-Munguía et al. 2004). This is in consistent with the blebs (probably encystation vesicles), I observed on the surface of the encysting *S. salmonis* (Fig. 5.3.1k). Exocytosis of the encystation vesicles would then allow assembly of the cyst wall, which – in the case of *Giardia* – is composed on interconnecting filaments containing peptides and carbohydrate moieties (De Souza 2006). Although the location of synthesis of encystation vesicles in *S. salmonis* is not yet known, we expect it to be deep within the cytoplasm, as is the case in *G. lamblia* (De Souza 2006, Lanfredi-Rangel et al. 2003).

5.3.13 Discussion: Minimum infective dose

The adhesion ability of trophozoites and cysts of *S. salmonis* may facilitate spread of them via adhering to equipment or foods. Furthermore, additional buoyancy given to clusters by gas bubbles and plant debris in water may allow the clusters to float, and hence enhance transmission. In *S. meleagridis*, which infect birds, the clusters of cysts are enclosed within thick mucus clots, which has significant implications for the transmission of *S. meleagridis* by adhere to boots or feeders, or floating to the water surface in contaminated drinkers (Wood and Smith 2005).

Clusters of *S. salmonis* cysts would also ensure the simultaneous ingestion of large number of cysts, presumably exceeding the minimum infective dose (MID) for initiating a new infection. In *S. meleagridis*, the simultaneous ingestion of large numbers of cysts enclosed in protective mucus increase the chance of successful transmission of the parasite, and influences the severity of the resulting disease (Wood and Smith 2005).

Although minimum infective oral dose has not been established for piscine diplomonads, the number of individual diplomonads in the colonies we observed (approximately 50) lies within the range for minimum infective dose for avian and mammalian diplomonads. The MID is known for several mammalian diplomonads, for *G. lamblia* is about 10 – 100 (Roxström-Lindquist et al. 2005), for *G. muris* is 10 (Stachen and Kunstyr 1983), and for *Spironucleus muris* is 1 (Stachen and Kunstyr 1983).

5.3.14 Discussion: Anti adhesion therapy

I suggest that transmission of the life cycle can be disrupted by mechanical and/or chemical break up of the adhesive colonies. The anti adhesion therapy is now well-discussed against bacterial infections. The alarming increase in drug-resistant bacteria makes a search for new alternative methods important. An attractive approach is the use of agents that interfere with the ability of the bacteria to adhere to tissues of the host, since such adhesion is one of the initial stages of the infectious process. The validity of this approach has been unequivocally demonstrated in experiments performed in a wide variety of animals, from mice to monkeys, and recently also in humans. The various approaches to anti-adhesion therapy include the use of receptor and adhesin analogs, dietary constituents, sublethal concentrations of antibiotics, and adhesin-based vaccines (see details in Ofek et al. 2003). Therefore, the anti-adhesive drugs, once developed, may serve as a new means to fight cluster formation in *S. salmonis*, and subsequently decrease minimum infection doses for initiating new infection in rainbow trout.

5.3.15 Recommendations

Since cultivation of *S. salmonis* in the newborn calf serum medium consistently resulted in large numbers of trophozoites attaching and undergoing encystment, this media can be used for production of large numbers of cysts. Our serendipitous observation of the link between heat stress prior to inoculation, and the subsequent marked acceleration of attachment and encystment, suggest that subjecting the trophozoites to heat stress prior to inoculation (30 min at 30 °C) can produce a high yield of cysts within 30 minutes.

A video microscopy can be used to monitor trophozoite motility, attachment, and cluster formation in culture. The method used by Hutchings and Ludu (2004) is a good example of a video microscopy, in which the microscope slides are cleaned in a chromic acid bath for at least 30 minutes and washed copiously with ultra-pure water prior to cell motility assays, then a 7-15 microliters of culture was placed under a 22x22 mm cover slip, and the cells are visualized using an Olympus BX60 fluorescent microscope equipped for differential interference contrast microscopy. For more details, see Hutchings and Ludu (2004).

For having a detailed structure of the trophozoites and cysts, the smears with cysts and trophozoites can be staining in two methods; with the rapid stain Hemacolor®Merk (VWR International), or Heidenhain Iron Hematoxylin (HIH), which has been usefully used on *S. meleagridis* from birds by Wood and Smith (2005). Hemacolor provides differential color staining of trophozoites and cyst, and HIH results in clear, more-detailed staining of cell structure (wood and Smith 2005).

For investigating the ultrastucture of cyst wall in *S. salmonis*, the preservation and visualization of the surface components of the cysts can be improved with the use of ruthenium red during osmium tetroxide fixation (Chávez-Munguía et al. 2004).

5.4 Pathology associated with *Spirotrunculus salmonis*

5.4.1 Result: Histopathology – entire cross section (H&E)

No inflammation or hemorrhage was seen in any infected or uninfected fish. Intra-epithelial cells (IEC's) were recognized as small dark cells, surrounded by a clear sometimes eosinophilic halo, and usually lying basally in the mucosal epithelial cells (Fig. 5.4.1); The IEC's were seen in 3 of 15 infected fish, and in 2 of 15 uninfected fish. In infected fish, the number of IEC's per cross section were 1, 3, and 17 (mean = 7). In uninfected fish, the numbers of IEC's per cross section were 2, and 2.

Examination of the mucosal epithelial cells did not show evidence of cytoplasmic blebbing, swelling at the tips of the mucosal epithelia cells, nor loss of microvilli in any fish.

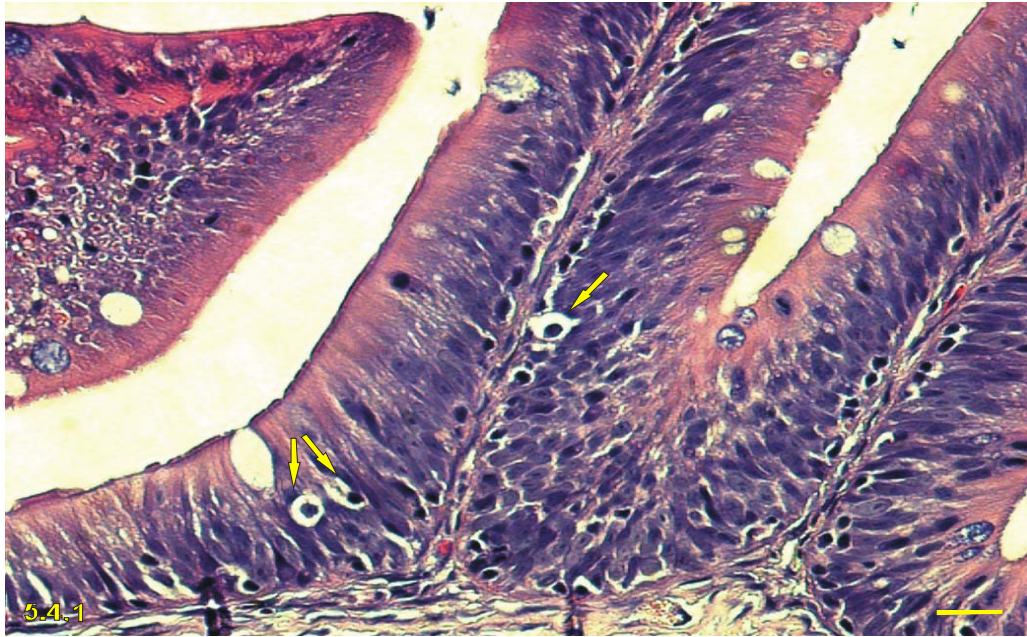


Fig. 5.4.1. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note intra-epithelial cells (IEC's) (arrows); the dark center surrounded by a clear halo. Hematoxylin and eosin. Scale bar = 20 μm .

5.4.2 Result: Histopathology - quantification of goblet cells (PAS/AB)

Hyperplasia: In infected fish, the number of “complete” goblet cells / 100 μm length of folds in the pyloric region ranged from 3 – 23 (mean = 8.5, SD = 2.7), and in uninfected fish the number ranged from 4 – 23 (mean = 9.4, SD = 2.4) (Table 5.4.1). These numbers were not significantly different between infected and uninfected fish, indicating that there was no hyperplasia of goblet cells. When numbers of “all” goblet cells were considered together, (i.e. “complete” and “putative” goblet cells), the numbers / 100 μm for infected fish were 5 – 26 (mean = 11.2, SD = 3.2), and for uninfected fish 5 – 27 (mean = 11.9, SD = 2.7); there was also no significant difference between infected and uninfected fish (Table 5.4.1).

Hypertrophy: In infected fish, the length of the goblet cells ranged from 7 – 25 μm (mean = 15.2, SD = 2.6), and in uninfected fish from 8 – 25 μm (mean = 14.2, SD = 1.2) (Table 5.4.1). These lengths were statistically significantly different. In infected fish, the width of the goblet

cells ranged from 4 – 19 μm (mean = 11.5, SD = 2.1), and in uninfected fish from 5 – 16 μm (mean = 9.9, SD = 1.3) (Table 5.4.1). These widths were statistically significantly different.

Hyperactivity: Hyperactivity of goblet cells was not evident in none of the uninfected fish (Fig. 5.4.2), but was observed in 1 of 15 infected fish (Figs. 5.4.3, and 5.4.4). In this infected fish, abundant mucus was being extruded from most of the goblet cells.

Table 5.4.1. Numbers, sizes, and activity of goblet cells in pyloric region of 15 juvenile rainbow trout infected with moderate density *Spironucleus salmonis*, and in 15 uninfected fish. Data are: top row, minimum and maximum values among the 75 individual measurements for number / 100 μm folds (15 fish, 5 folds per fish), and among the 225 individual measurement for size (15 folds, 5 folds per fish, 3 largest goblet cells per fold); middle row, minimum and maximum values among the 15 means; bottom row, mean \pm SD for all 15 fish. ns, not significantly different by unpaired t- test.

Parameter	Infected fish	Uninfected fish	Results
Number / 100 μm fold			
All goblet cells ^a	5 – 26 7.3 – 16.8 11.2 \pm 3.2	5 – 27 8.1 – 19.1 11.9 \pm 2.7	ns
Complete goblet cells ^b	3 – 23 4.8 – 13.2 8.5 \pm 2.7	4 – 23 6.9 – 16.4 9.4 \pm 2.4	ns
Size ^c			
Length	7 – 25 10.3 – 18.6 15.2 \pm 2.6	8 – 25 11.9 – 16.1 14.2 \pm 1.2	significantly different ^d
Width	4 – 19 8.2 – 14.7 11.5 \pm 2.1	5 – 16 7.5 – 13.0 9.9 \pm 1.3	significantly different
Hyperactivity	1 of 15	none	test not done

^a All goblet cells = complete and putative

^b Complete goblet cells are those for which the complete goblet-shaped profile could be clearly seen in longitudinal section

^c Length and width measured at the largest bowl-shaped part of the cell, opening onto the cell surface

^d Two outlier points removed from analysis



Fig. 5.4.2. Histological section of pyloric region of the digestive tract of juvenile rainbow trout *Oncorhynchus mykiss*. Note normal activity of goblet cells, and the typical shape of the goblet cells, with a distinct distal bulb, and in most cells the proximal stem is rather narrow, if it can be seen at all. Note also that there is very little mucus being exuded beyond the surface of the enterocytes. Combined PAS / AB (alcian blue). Scale bar = 40 μm .

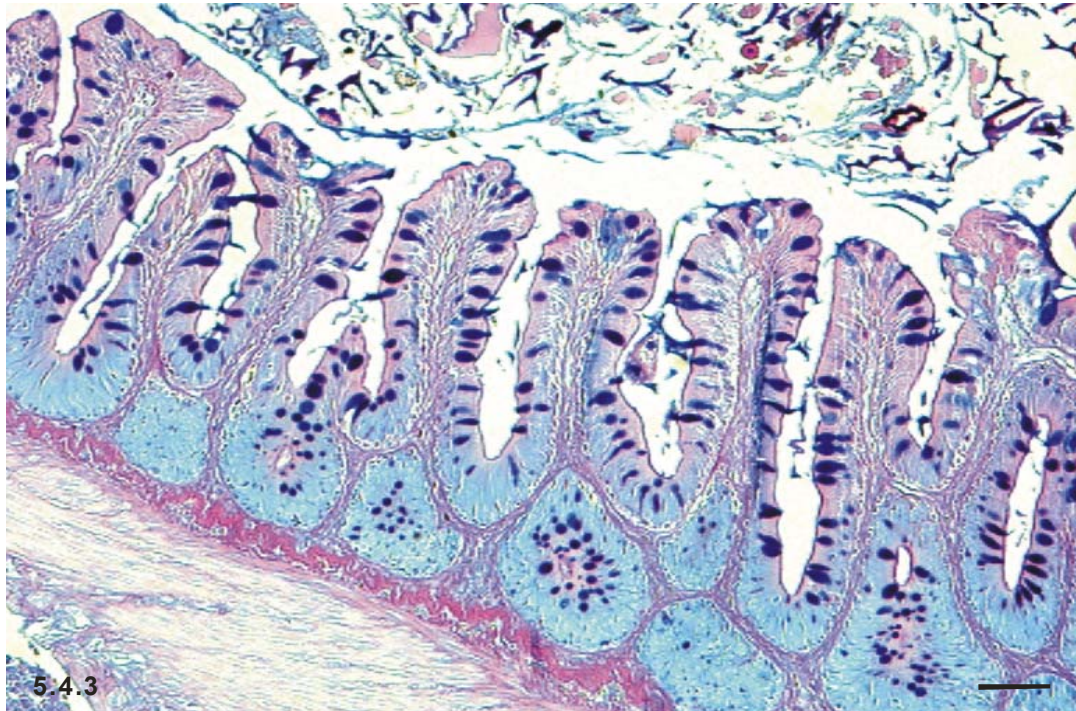


Fig. 5.4.3. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note the hyperactivity of goblet cells. Note also that the distal goblets are dilated, the proximal stems are dilated and easily seen in most cells, and that abundant mucus is being exuded above the enterocytes. Combined PAS / AB (alcian blue). Scale bar = 40 μ m.

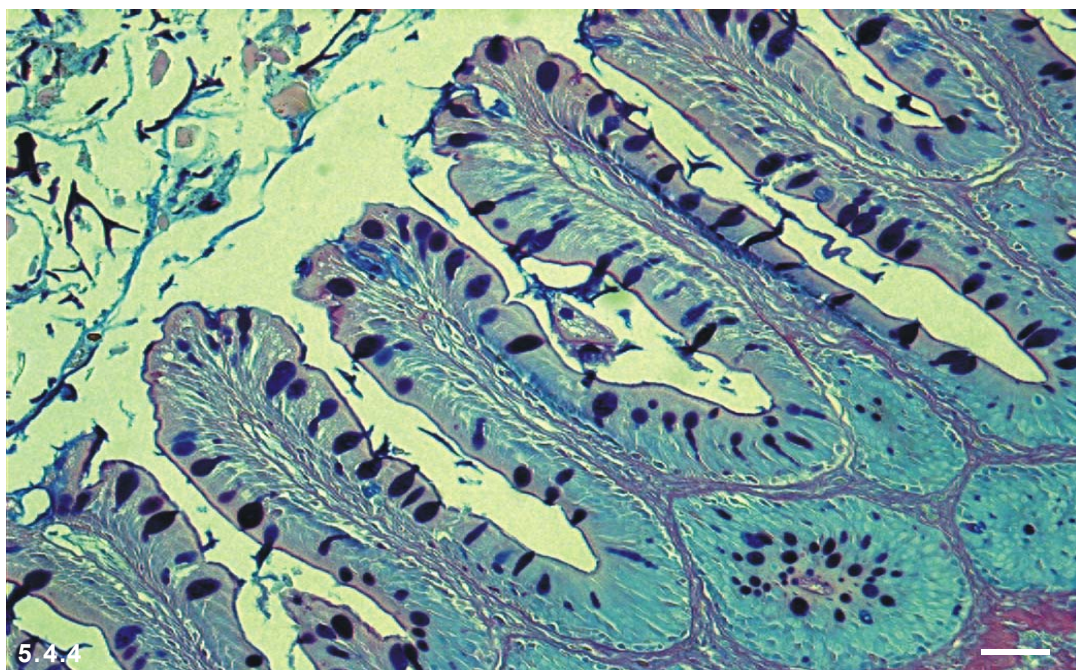


Fig. 5.4.4. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note the hyperactivity of goblet cells. Lower magnification of the section in Fig. 5.4.3, to show the extent of the hyperactivity. Combined PAS / AB (alcian blue). Scale bar = 80 µm.

5.4.3 Result: Histopathology – new symptoms

Five other types of pathology were seen: vacuolization, clubbing, loss of fold architecture, shortened folds, and crowded nuclei in the mucosal epithelium (Table 5.4.2).

Table 5.4.2. Occurrence and severity of diverse pathology in pyloric region of 15 juvenile rainbow trout *Oncorhynchus mykiss* infected with moderate density *Spironucleus salmonis*, and in 15 uninfected fish. Data presented is: top row, number of affected fish; bottom row, minimum and maximum and mean % of cross section that was affected.

Parameter	Infected fish	Uninfected fish	Results for occurrence
Vacuolisation	5 5 – 25, 11	0	significantly different
Clubbing	10 5, 5	9 5, 5	n s
Loss of fold architecture	15 5 – 15, 6	13 5 – 10, 5	n s
Shortened folds	15 5 – 25, 6	15 5, 5	n s
Crowded epithelial nuclei	8 5, 5	5 5, 5	n s

Vacuolisation of the enterocytes was seen in significantly more infected than uninfected fish, affecting one third of the *S. salmonis* infected fish (Fig. 5.4.5), but none of the uninfected fish. In the affected fish, vacuolization affected 5 – 25% of the surface area seen in the cross

sections. Clubbing was seen in approximately two thirds of infected and uninfected fish, and affected approximately 5% of their fold area (Fig. 5.4.6). Shortened folds, and loss of fold architecture were each also seen in infected and uninfected fish, affecting some 5 – 25% of the fold area (Fig. 5.4.6, and Fig. 5.4.7, respectively). Crowded epithelial nuclei were seen in approximately one third of infected and uninfected fish, affecting some 5% of the fold area (Fig. 5.4.8).



Fig. 5.4.5. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note vacuolization affecting most of the mucosal epithelial cells. Hematoxylin and eosin. Scale bar = 40 μ m.

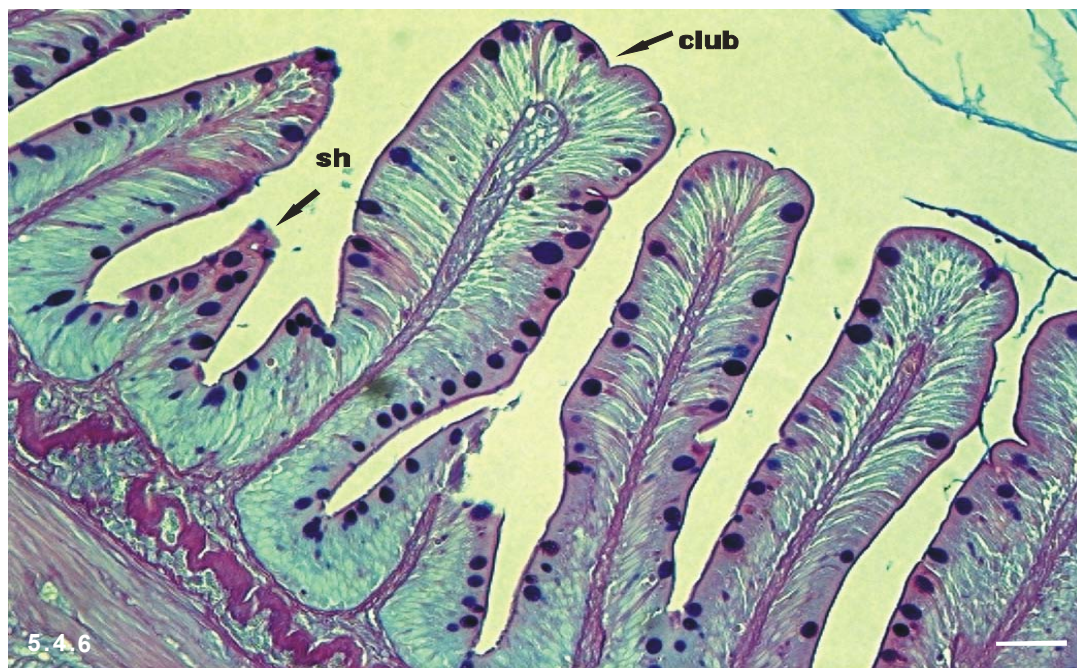


Fig. 5.4.6. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note clubbing (club) and shortening (sh) of a fold. Combined PAS / AB (alcian blue). Scale bar = 40 μ m.

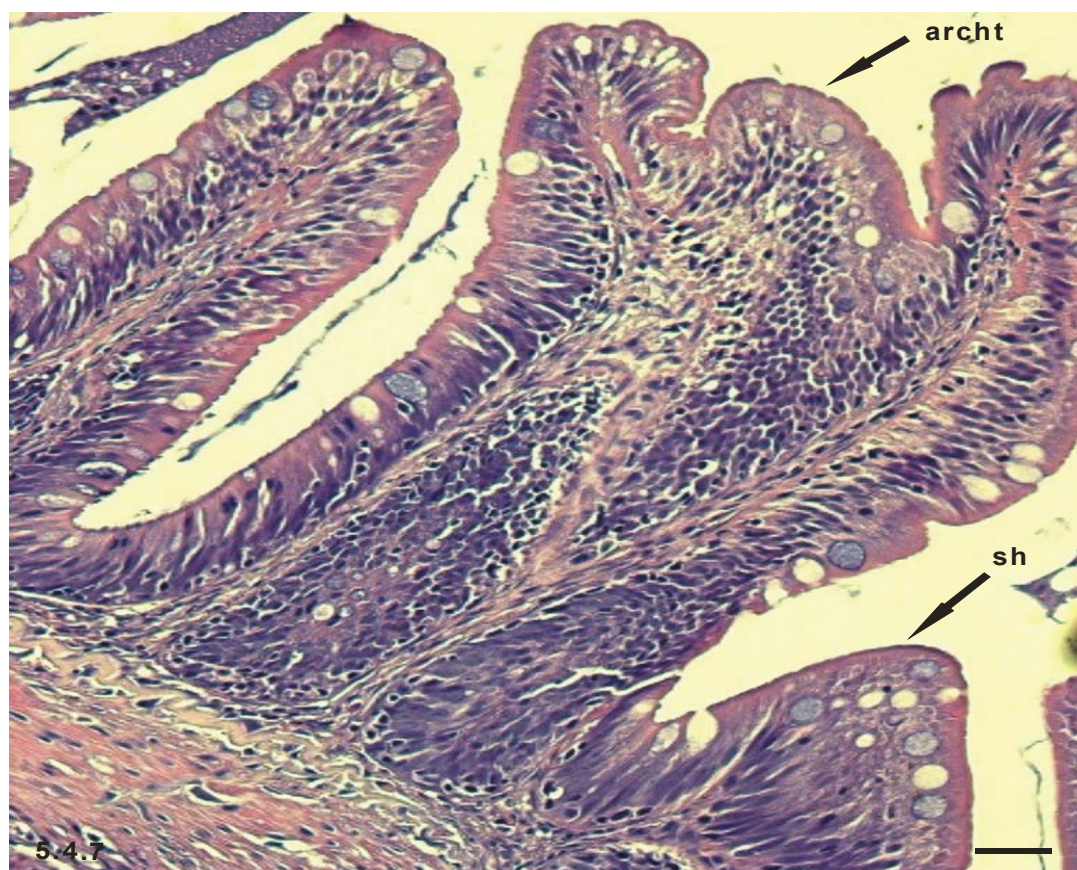


Fig. 5.4.7. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note fold architecture (archt) and shortening (sh) of a fold. Hematoxylin and eosin. Scale bar = 40 µm.

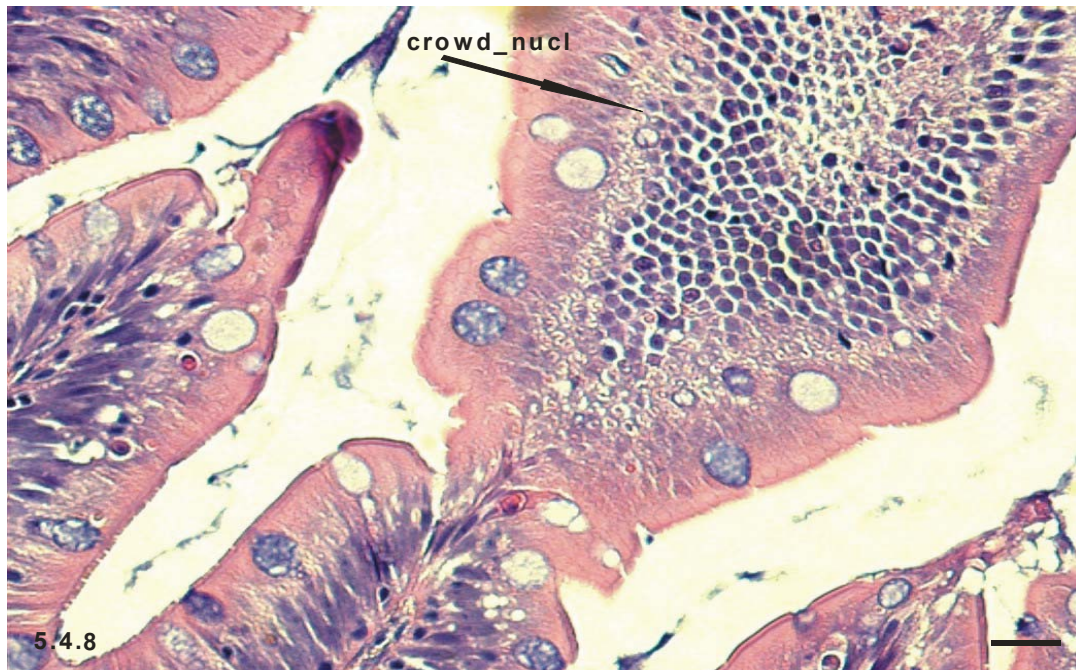


Fig. 5.4.8. Histological section of pyloric region of the digestive tract of infected juvenile rainbow trout, *Oncorhynchus mykiss* with moderate density *Spironucleus salmonis*. Note crowded nuclei. Hematoxylin and eosin. Scale bar = 20 µm.

Liver: There was no hepatocellular necrosis seen in any fish.

5.4.4 Discussion: Overview

Although based on the present study, I believe now that cytoplasmic blebbing is artefact, a variety of pathologies were observed in the pyloric region of the fish, the most marked of which was the significant hypertrophy and hyperactivity of goblet cells in the infected fish when compared to the uninfected fish; there was no evidence of goblet cell hyperplasia. The present finding of hypertrophy and hyperactivity of goblet cells in *S. salmonis* infected fish,

even in those without apparent clinical signs, highlights that the disease associated with the infection is probably multi-factorial.

5.4.5 Discussion: Goblet cells and their possible relationship with pathology

A mechanism for the increased mucus production in *S. salmonis* infected rainbow trout has now been identified, with the documentation of significant hypertrophy. Although the amount of mucus produced by individual goblet cells increased, as evident by both increased size and activity, the number of cells did not increase. Increased mucus production may decrease nutrient absorption, and underlie the impaired growth in *S. salmonis* infected rainbow trout. These mechanisms do not appear to have previously been recognized. Ferguson (1979) noted excess mucus, but did not report changes in the appearance and activity of the goblet cells that could be responsible for the excess mucus. Sano (1970) reported catarrhal enteritis.

We suggest that additional mechanisms of pathogenicity need to be sought in piscine diplomonads, for example impaired absorption and altered haematology and biochemistry have recently been reported in spironucleosis in game birds (Lloyd et al. 2005), and similar problems might pertain in spironucleosis in fish. Key aspects of the potential pathogenicity of enteric piscine diplomonads have probably been overlooked in previous studies, because of the lack of consideration of comparative pathobiology of *Spironucleus* infections in non-piscine hosts.

In the present study the significant hypertrophy of the goblet cells in *S. salmonis* infected fish was demonstrated, yet no such pathology was seen in similar sized uninfected fish. Whether the hypertrophy occurs in response to the diplomonad infection, or is simply concurrent with it, we are unable to determine. To resolve this question, a future investigation could be undertaken with comparison of experimentally infected fish that had not previously been exposed to *S. salmonis*, and their uninfected cohorts.

Although we documented evidence of excess mucus in *S. salmonis* infected fish, there was no evidence of inflammation and or hemorrhage, two conditions previously reported as associated with *S. salmonis* infection. This difference may be attributed to the different ages and presentations of the fish in our study compared with previous studies. In my investigation, the focus was on juvenile fish 5 – 6 months old, and with a mean length of 12 cm, none of which showed clinical signs of *S. salmonis* infection. This group of fish was chosen for study, as they are the focus of numerous investigations in my experiments. In contrast, most previous studies of *S. salmonis* infection have focused on younger, smaller fish, such as fry and fingerlings, in which clinical signs are most commonly seen. A further compounding factor is that I can not be certain that my uninfected fish has never been exposed to *S. salmonis*, I could only show that they were not infected at the time of harvesting the tissues.

The present finding of hypertrophy and hyperactivity of goblet cells in *S. salmonis* infected fish, even in those without apparent clinical signs, highlights that the disease associated with the infection is probably multi-factorial.

5.4.6 Discussion: What are intra-epithelial cells?

The identity of the intra-epithelial cells seen in *S. salmonis* infected trout was unknown to Ferguson (1979) and remains so. He suggested they were apoptotic cells or represented shrinkage necrosis, as seen in both normal and diseases tissues, and considered it unlikely that they were intra-cellular forms of *S. salmonis*. His observation that these cells contained mitochondria would rule out their identity as *S. salmonis*, since diplomonads do not have typical mitochondria, but rather mitosomes (Tovar et al. 2003). We observed a higher density of these cells in the infected trout compared with the uninfected trout; however the significance of this is not clear.

5.4.7 Discussion: Is cytoplasmic blebbing artifact?

The present study has helped to resolve the dilemma concerning the appearance of “cytoplasmic blebbing” in *S. salmonis* infected rainbow trout. Ferguson (1979) suggested that this was fixation artifact, and my study is in agreement with this. Although he observed this leakage of cytoplasm by scanning and transmission electron microscopy, I am confident I would have seen it in the light microscopy study had it been present, since examination of the 3 µm sections at 400x allowed us a resolution of 1 µm, which was considerably smaller than the blebs.

5.4.8 Implications

If further studies support the hypothesis that hypertrophy and hyperactivity of goblet cells are associated with *S. salmonis* infection, this suggests a new treatment option, namely administration of treatment to reduce mucus production. This provides a novel solution to the present problem, where the only effective drugs are the metronidazole, which kill the *S. salmonis* trophozoites, are not permitted for use in food fish in many countries, including much of Europe (Buchmann and Bresciani 2001), and to which resistance has been reported (Tojo and Santamarina 1998).

5.4.9 Recommendations

It is recommended that hyperplasia, hypertrophy and hyperactivity of goblet cells in the pyloric region be investigated in naturally infected fry and fingerlings using the new quantitative protocol we now present. We also recommend that these parameters be investigated in experimentally infected fish, and their uninfected cohorts, to try to determine if *S. salmonis* is the cause of the changes in the goblet cells, or simply correlated with them.

5.5 *In vitro* plasma incubation test – host susceptibility to *S. salmonis*

5.5.1 Result: Developing *in vitro* plasma incubation test – refining protocol

Density of trophozoites in the well: A total of 20-40 trophozoites in 50 µl culture in the well were the most suitable combination for the 384 well plates. The motile trophozoites could easily be counted.

Plasma – serial dilutions: Generally, the survival of the trophozoites decreased when the plasma concentration increased (Fig. 5.5.1). The percentage of survival of the trophozoites decreased at each plasma concentration with time, for concentrations > 1:512 (Fig. 5.5.1). For rainbow trout, there was no significant differences in survival of trophozoites between 3 concentrations (1:512, 1:1024, 1:2048) and control (ANOVA, $p > 0.05$) (Fig. 5.5.1).

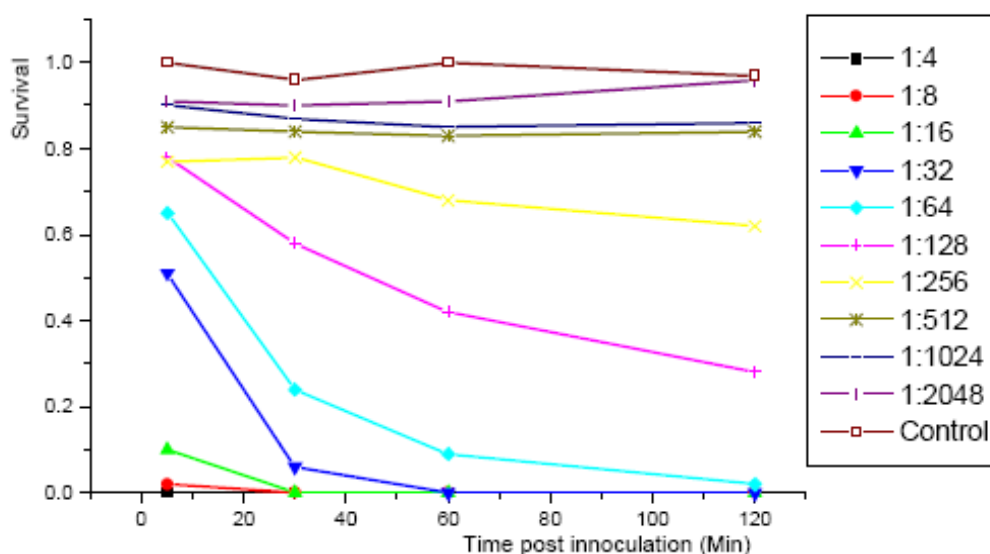


Fig. 5.5.1. The effect of different rainbow trout plasma concentrations on survival of trophozoites monitored over 120 min ($n=3$), survival scale 1.0 = 100% survival (from Cheng 2006).

Time check points: The rapid changes in the number and behaviour of the trophozoites were observed within the first 5 min of incubation, thus monitoring the cultures should begin at 5 min of inoculation. In rainbow trout plasma, few trophozoites were still alive 120 min after incubation, thus all 4 time check points (5, 30, 60, 120 min) should be considered for *in vitro*

plasma of rainbow trout. However, 3 time check points (5, 30, 60 min) was enough for monitoring diplomonads in carp and sturgeon plasma, since no trophozoites were alive after 60 min incubation in their plasma.

The trophozoites survival in carp plasma was higher than that in Atlantic sturgeon plasma at each concentration and time point (Figs 5.5.2, 5.5.3).

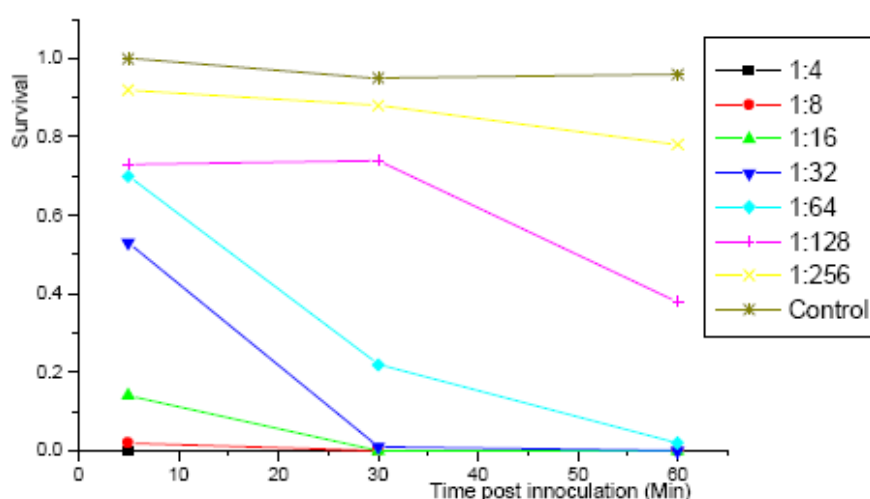


Fig. 5.5.2. The effect of different carp plasma concentrations on survival of trophozoites monitored over 60 min (n=2), survival scale 1.0 = 100% survival (from Cheng 2006).

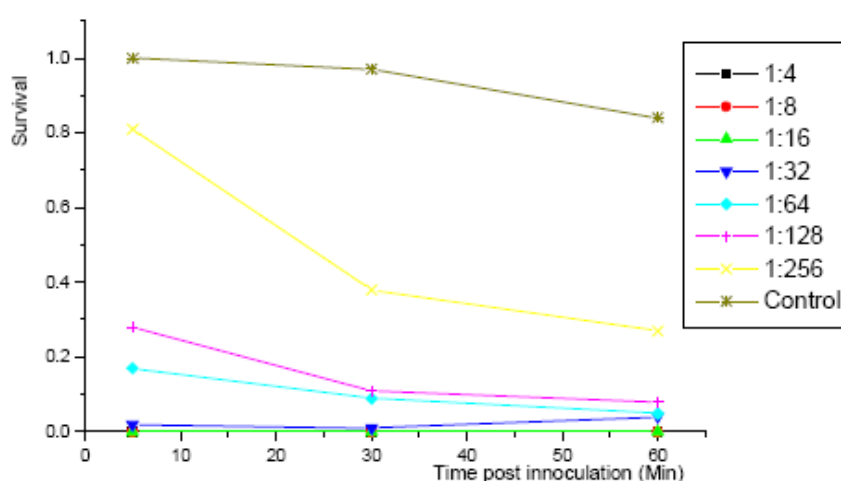


Fig. 5.5.3. The effect of different sturgeon plasma concentration on survival of trophozoites monitored over 60 min (n=3), survival scale 1.0 = 100% survival (from Cheng 2006).

Detecting lysis and cytotoxicity: The number of the trophozoites at each time check was subtracted from the number of trophozoites in the control well; thus the result was the lysis of trophozoites in each time check point.

Normal movement of trophozoites in the control wells was quickly and freely active. However the following abnormalities in movement were observed in plasma concentrated wells; 1) swimming very slowly and shaking, but still forward, 2) stay in one point and turning around or shaking, 3) completely immobilized. The number of trophozoites with any of the above abnormalities was counted as the cytotoxicity parameter for each well.

Replications: Triplicate test for each individual fish were conducted, with three control wells for each replicate; this represented a reasonable compromise considering the fact that the protocol was time consuming (time for plate reading), and considering the challenges of counting the motile trophozoites.

5.5.2 Result: The new *in vitro* plasma incubation test protocol

First of all, 25 µl of culture medium (without trophozoites) was loaded into each well of a 384 well plate (maximum 13 wells for 10 serial plasma concentrations and 3 control wells). Then 25 µl of plasma was dispensed into the first well, and serial dilutions were made (for rainbow trout the serial dilution was from 1:4 to 1:2048 (10 wells); for carp and sturgeon, the dilution was from 1:4 to 1:256 (7 wells); the 3 control wells were without plasma). Finally, each of 13 wells was inoculated with 25 µl of the culture medium containing of trophozoites (20-40 trophozoites / 25 µl medium). The 25 µl medium was inoculated in the order from the control well to most concentrated plasma well, to reduce the impact of contamination by accidental transfer of plasma. The final volume in each well plate was 50 µl. Well plate was held at 10°C in dark place for up to 120 min, using a cool box with ice and thermometer.

At each time check point (5, 30, 60, 120 min for rainbow trout, and 5, 30, 60 min for carp and sturgeon) the well plate was monitored under the inverted microscope, in order to record

numbers of normal and abnormal swimming trophozoites. Normal swimming was free, active movement, and abnormal swimming included slow movement, shaking, and turning on the spot.

Lysis and cytotoxicity were calculated as follows; lysis is the initial number of normal and abnormal trophozoites (control) minus the number of normal and abnormal trophozoites at 5, 30, 60 or 120 min. Cytotoxicity is the number of abnormal trophozoites at each time point.

5.5.3 Result: Using new plasma incubation test to predict host susceptibility

Lysis

Lysis: effect after 5 min plasma incubation: In rainbow trout and carp, the lowest plasma concentration (less plasma in culture) that could cause 100% lysis (all diplomonads dead) was at plasma concentration 1:4. In sturgeon, the lowest plasma concentration that could cause 100% lysis was recorded at plasma concentration 1:16; thus the sturgeon plasma had a stronger lytic affect in lower plasma concentration than rainbow trout plasma and carp plasma (Fig. 5.5.4).

After 5 min plasma incubation, in all 3 fish species, the lytic effect of plasma increased when the plasma concentration increased – more plasma in culture, more lysis (Fig. 5.5.4).

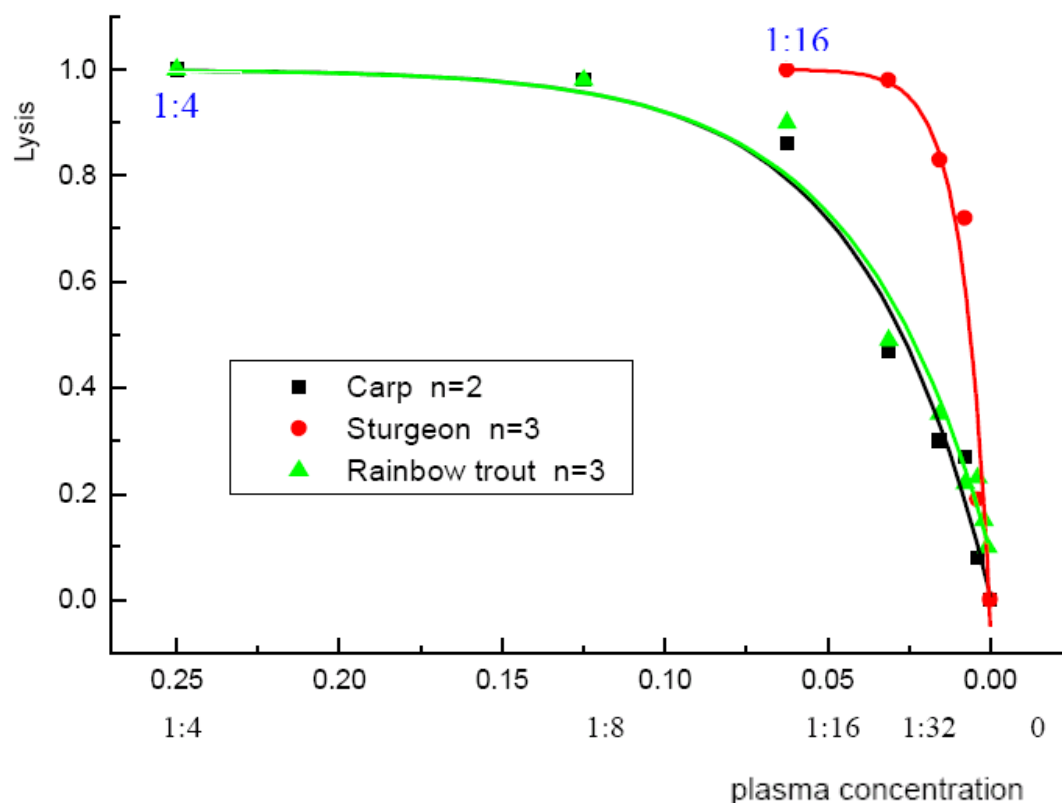


Fig. 5.5.4. The lysis affect of rainbow trout plasma (n=3), carp plasma (n=2), and sturgeon plasma (n=3) on *S. salmonis* trophozoites in different plasma concentration, and after 5 min plasma incubation at culture (*in vitro* plasma incubation test), lysis scale 1.0 = 100% lysis. Graph was prepared by Ms Cheng, M.Sc. student from Humboldt University of Berlin.

Lysis: effect after 30 min plasma incubation: In rainbow trout, carp and sturgeon, the lowest plasma concentration that could cause 100% lysis affect was the same and recorded at plasma concentration 1:16. However, in lower plasma concentration than 1:16 (i.e. 1:64, and 1:128), the sturgeon plasma showed stronger lysis affect than the rainbow trout plasma, and carp plasma (Fig. 5.5.5).

After 30 min plasma incubation, in all 3 fish species, the lytic affect of plasma increased when the plasma concentration increased – more plasma in culture, more lyses (Fig. 5.5.5).

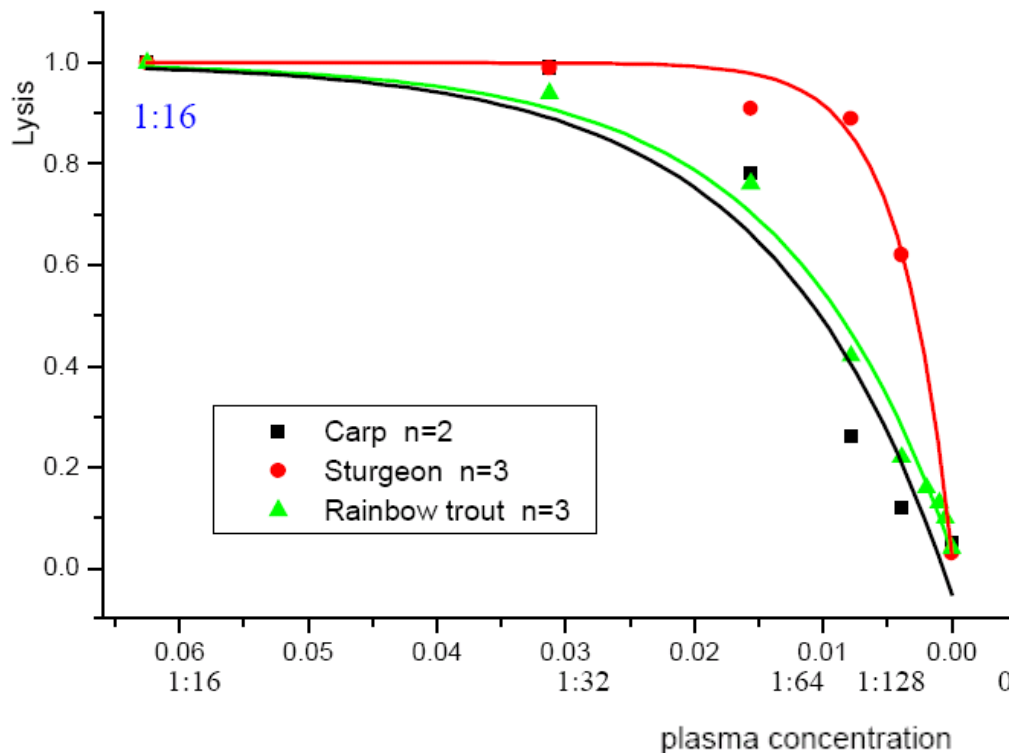


Fig. 5.5.5. The lysis affect of rainbow trout plasma (n=3), carp plasma (n=2), and sturgeon plasma (n=3) on *S. salmonis* trophozoites in different plasma concentration, and after 30 min plasma incubation at culture (*in vitro* plasma incubation test), lysis scale 1.0 = 100% lysis. Graph was prepared by Ms Cheng, M.Sc. student from Humboldt University of Berlin.

Lysis: affect after 60 min plasma incubation: In rainbow trout and carp, the lowest plasma concentration that could cause 100% lysis was recorded at plasma concentration 1:32. In sturgeon, the lowest plasma concentration that could cause 100% lysis was at plasma concentration 1:16 (Fig. 5.5.6). In lower plasma concentrations, the sturgeon plasma showed stronger lysis affect than rainbow trout plasma, and carp plasma (Fig. 5.5.6).

After 60 min plasma incubation, in all 3 fish species, the lysis affect of plasma increased when the plasma concentration increased – more plasma in culture, more lysis.

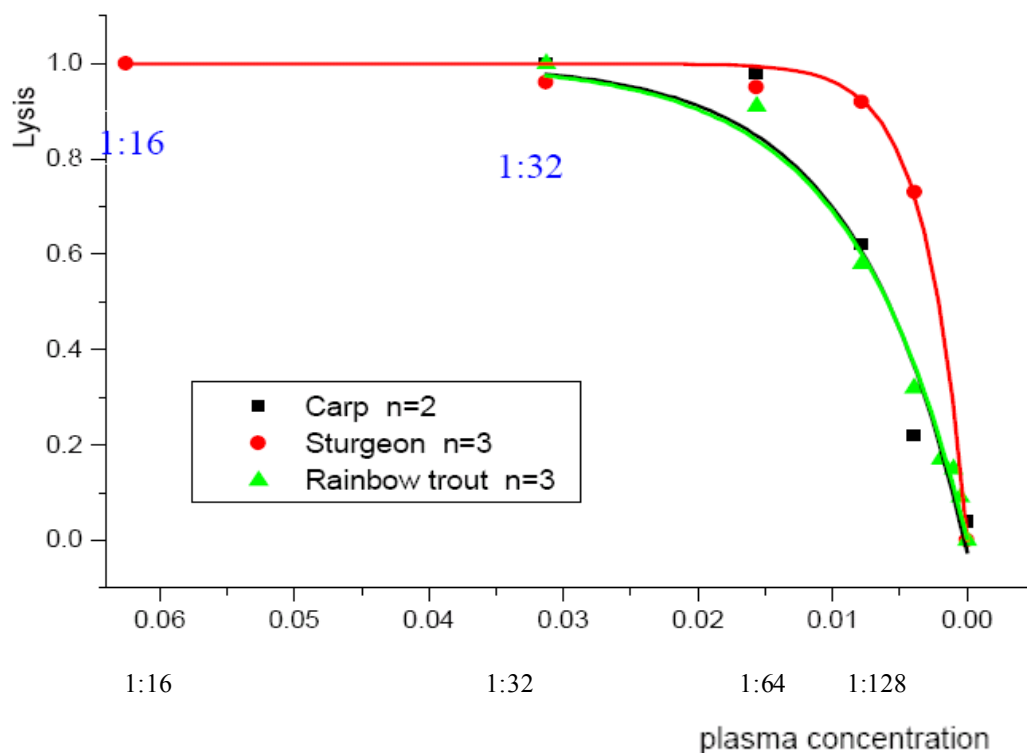


Fig. 5.5.6. The lysis affect of rainbow trout plasma (n=3), carp plasma (n=2), and sturgeon plasma (n=3) on *S. salmonis* trophozoites in different plasma concentration, and after 60 min plasma incubation at culture (*in vitro* plasma incubation test), lysis scale 1.0 = 100% lysis. Graph was prepared by Ms Cheng, M.Sc. student from Humboldt University of Berlin.

In all 3 fish species, and in all time check points, the increase of plasma concentration increased lytic affect of plasma – more plasma in culture, more lysis.

Rainbow trout plasma and carp plasma showed the same lytic affect in all plasma concentrations, and in all time check points. The sturgeon plasma had stronger lytic affect in lower plasma concentrations in all time check points than rainbow trout plasma and carp plasma.

When the time post-incubation increased, the lytic affect of rainbow trout plasma and carp plasma increased – stronger % lysis in lower plasma concentrations. The sturgeon plasma was so strong that the increase of time post-incubation had no affect on the lytic ability (the lowest plasma concentration that could cause 100% lysis was 1:16 at all time check points).

The percentage of lysis in rainbow trout and carp plasma had a gradual increase (for example from 0 to 10% to 30% to 50%, and so on) in all time check points, when the plasma concentrations increased (for example from....1:32 to 1:16 to 1:8, and to 1:4),. However, in sturgeon plasma, the percentage of lysis increased very quickly from zero to 100% by increasing the plasma concentrations.

Cytotoxicity

Cytotoxicity affect after 5 min plasma incubation: In rainbow trout and carp, the percentage of cytotoxicity (abnormally swimming trophozoites) increased when the plasma concentration increased (Fig. 5.5.7). The cytotoxic affect in rainbow trout plasma was stronger than that of carp plasma at each plasma concentration (Fig. 5.5.7).

In sturgeon, there were few data points, and they were very scattered, so no relationship was seen.

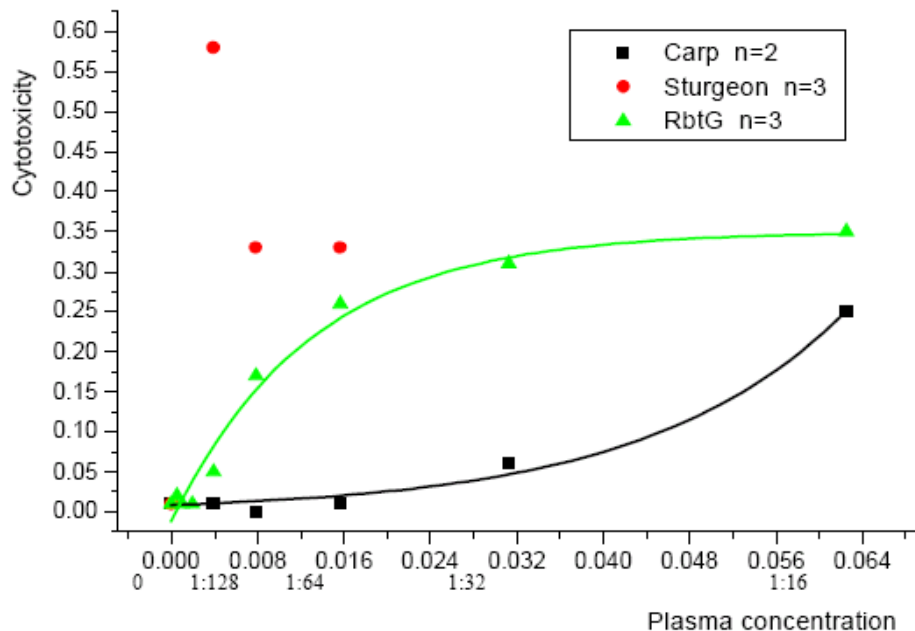


Fig. 5.5.7. The cytotoxicity affect of rainbow trout plasma (n=3), carp plasma (n=2), and sturgeon plasma (n=3) on *S. salmonis* trophozoites in different plasma concentration, and after 5 min plasma incubation at culture (*in vitro* plasma incubation test), cytotoxicity scale

0.60 = 60% cytotoxicity. Graph was prepared by Ms Cheng, M.Sc. student from Humboldt University of Berlin.

Cytotoxicity: affect after 30 min plasma incubation: In rainbow trout and carp, the percentage of cytotoxicity increased when the plasma concentration increased (Fig. 5.5.8). The cytotoxic affect in rainbow trout plasma was stronger than that of in carp plasma at each plasma concentration (Fig. 5.5.7).

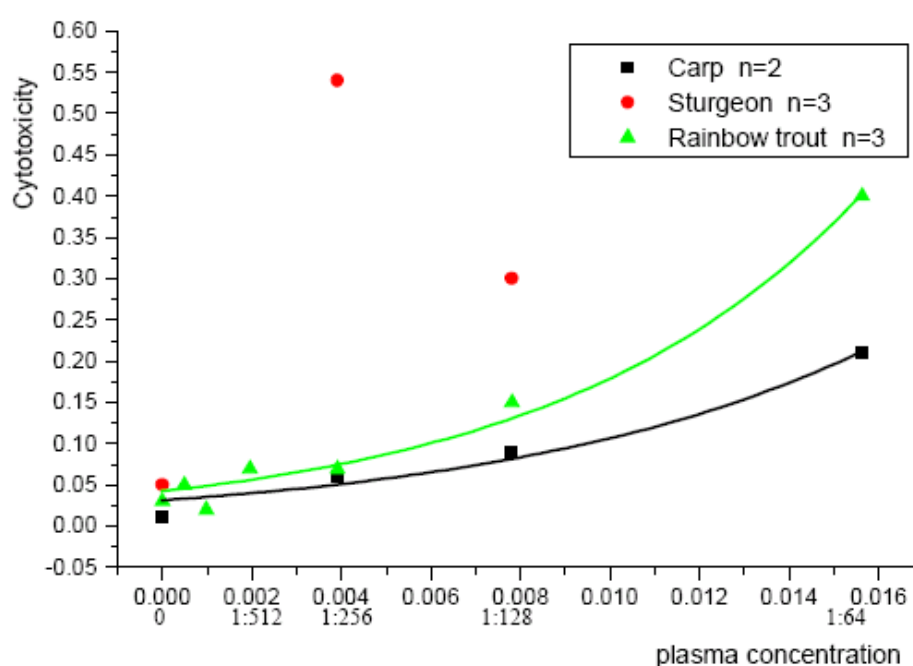


Fig. 5.5.8. The Cytotoxicity affect of rainbow trout plasma (n=3), carp plasma (n=2), and sturgeon plasma (n=3) on *S. salmonis* trophozoites in different plasma concentration, and after 30 min plasma incubation at culture (*in vitro* plasma incubation test), cytotoxicity scale 0.60 = 60% cytotoxicity. Graph was prepared by Ms Cheng, M.Sc. student from Humboldt University of Berlin.

Cytotoxicity affect after 60 min plasma incubation: In rainbow trout and carp, the percentage of cytotoxicity (abnormally swimming trophozoites) increased when the plasma concentration increased (Fig. 5.5.9). The cytotoxic affect in rainbow trout plasma was stronger than that of carp plasma at most plasma concentrations (Fig. 5.5.9).

In sturgeon, there were few data points, and they were very scattered, so no relationship was seen.

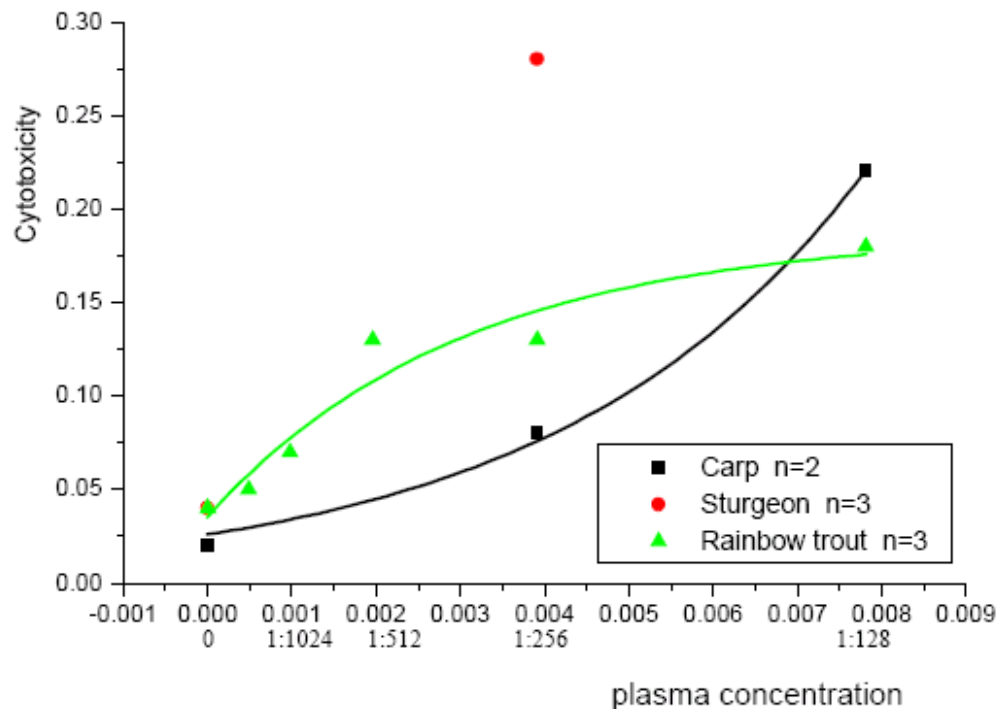


Fig. 5.5.9. The cytotoxicity affect of rainbow trout plasma (n=3), carp plasma (n=2), and sturgeon plasma (n=3) on *S. salmonis* trophozoites in different plasma concentration, and after 60 min plasma incubation at culture (*in vitro* plasma incubation test), cytotoxicity scale 0.30 = 30% cytotoxicity. Graph was prepared by Ms Cheng, M.Sc. student from Humboldt University of Berlin.

5.5.4 Discussion: Overview

The present study demonstrates that the *in vitro* plasma incubation test used for the piscine kinetoplastid heamoflagellate *Cryptobia*, and the mammalian enteric diplomonad *Giardia* can be successfully modified for the enteric piscine diplomonad *Spironucleus*. An essential requirement was demonstrating that the response of the diplomonads was dependant on the concentration of the plasma (dose-response), and this we demonstrated. The most significant modifications were adding abnormal motility as a new parameter for recording the cytotoxicity affect of plasma, and adding extra time check points to monitoring the dynamics

of the lysis and cytotoxicity affect of plasma in post-incubation times.

The plasma from Atlantic sturgeon, not reported as a host for diplomonads, showed stronger lytic and cytotoxic effects than the plasma of rainbow trout and carp; thus the hierarchy of the lytic and cytotoxic effects of the plasma to *S. salmonis* was: Atlantic sturgeon > rainbow trout and carp. Between the last two species, the diplomonads could survive for a shorter time in the plasma of carp, than in plasma of rainbow trout. Thus, the hierarchy of severity of the effects of the plasma was: sturgeon > carp > rainbow trout. The modified plasma incubation test may demonstrate an aspect of the parasite-host relationship, namely host susceptibility, which mirrors aspects of the epizootiological results (what is known from natural host-parasite records).

5.5.5 Discussion: Refining the plasma incubation protocol for *S. salmonis*

Establishing a stable culture medium for *S. salmonis* is the most important and essential part for the experiment. The culture protocol, which have been previously used for culturing diplomonads from rainbow trout (presumably *S. slamonis*) suggested by Buchmann and Uldal (1996) were not consistently successful in my experiment.

The optimal pH for culturing *S. salmonis* was suggested as between 7.5 to 8 by Buchmann and Uldal (1996). The pH of 8.0 was not good for the *in vitro* culture of *S. salmonis* in my experiment. Generally, the cultures were successful at pH 7.5, and my study on microhabitat preference of diplomonads *in vivo* has shown that the pH of 7.3 is the real pH preferred by *S. slamonis*; thus I strongly suggest that the further culture be established at pH 7.3, since this is the pH of preference for *S. salmonis* in intestine of rainbow trout.

I also found that the heavily infected fish are the most successful density to establish the culture. The contents of the pyloric region from 3 heavily infected rainbow trout together in approximately 45 ml of culture medium in a 50 ml plastic culture flask can give a good chance for a successful culture (see section 4.5.1 “*In vitro* culture”).

There were some difficulties to get enough heavily infected fish during summer 2006. The occurrence and density infection were decreased significantly, and remained low, most infections were light, and some were moderate. The other locations in Germany reported the same. The lower infection in farm was probably related to the unusual warm weather in summer 2006, which was the warmest and longest in the last decade. *Spironucleus salmonis* is very temperature sensitive, *in vitro* studies show that the number of parasite declines significantly at temperatures > 15 - 20 °C (Buchmann and Uldal 1996). In the other hand, stock source of *S. salmonis* in culture were not available from ATCC (American Type Culture Collection), or from any other known source.

The chance for establishing diplomonad culture using Buchmann and Uldal (1996) protocol was about 30% in my experiment, and the alternative culture medium suggested by Uldal (1996) were also not useful for the present study, since it produced more cyst than trophozoites in culture.

Although most of the parameters that have been modified for successful plasma incubation test on *S. salmonis* are compatible for other piscine diplomonads, some parameters such as the temperature needs to be changed based on diplomonad species, for example *S. vortens* needs 25 °C for *in vitro* culture (Poynton et al. 1995), and this needs temperature should be set for *in vitro* plasma incubation test on this specific diplomonad species.

The quality of the trophozoites in the *in vitro* culture of *S. salmonis* or other diplomonad species can influence the accuracy of the lysis and cytotoxicity data. A poor quality culture (diplomonads for example swimming slowly, with shaking), will cause inaccuracy on the data of cytotoxicity, and the number of dead diplomonads are less reliable for recording the lytic affect of the plasma, because the diplomonads are already compromised before exposure to the test plasma.

A more standardised density of trophozoite in well plates (to reduce the variation of 20-40 trophozoites / 25 µl) will give more accurate data records for lytic affect of plasma. Generally the numbers of small organisms like diplomonads or cells can be counted by

haemocytometer, however since diplomonads in culture are very active, it is difficult to count them in the haemacytometer chambers; thus the other method needs to be developed. Different serial dilutions from 1:4 to 1:2048 were tested for all three fish species; rainbow trout, carp, and sturgeon, to find out the appropriate range of serial dilutions of plasma (e.g. testing which plasma concentration has similar effect as the control with no plasma).

The time check points used to monitor the lysis affect of plasma from different resistant fish species may differ from each other, and it needs to be tested before performing the *in vitro* plasma incubation test. Some resistant fish species have a very strong lytic affect that can lyse pathogens in a shorter period of time in the same plasma concentration than other resistant fish.

The number of fish in each species should be more than 10 fish, to get a robust data set to determine if there is a significant result of *in vitro* plasma incubation test. Most of the time spent in the present study was focused on protocol development, and there was only limited time to test more fishes in each species, when trophozoites were available in culture.

The age, size, and sex of fish may also effect host susceptibility to the pathogen, and need to be considered when performing *in vitro* plasma incubation test. To avoid the influence of these factors, the fish can be chosen in the same age, size, sex, and sexual maturation, and investigators can perform the experiment in one season.

5.5.6 Discussion: Predict host susceptibility to *S. salmonis*

The modified plasma incubation test has been successfully used to detect lytic and cytotoxic affects of plasma from susceptible host (rainbow trout) and resistant hosts (common carp and Atlantic sturgeon) on piscine enteric *S. salmonis*. The test showed the following hierarchy of the lytic and cytotoxic effects of plasma on *S. salmonis*: sturgeon > rainbow trout and carp. Part of this was in agreement with epizootiological data, namely that Atlantic sturgeon, *A. sturio*, are not known to be hosts for any diplomonads, however it should be noted that other sturgeon species have been reported to be hosts for diplomonads - *A. ruthenus*, *A.*

gueldenstaedtii, *A. stellatus* from Russian were reported to carry diplomonads in gall bladder (Bauer et al. 2002). The marked lytic affect of plasma from Atlantic sturgeon shows that it may be resistant to *S. salmonis*.

The lysis affect of plasma from rainbow trout and carp was very close. The lowest plasma concentration (the weakest concentration of plasma in culture) that lysed 100% diplomonads in well plates was similar in both rainbow trout and carp plasma in all time check points (1:4 in 5 min, 1:16 in 30 min, 1:32 in 60 min). However, trophozoites could survive for a shorter time in the plasma of carp than of rainbow trout, suggesting that carp plasma may be less tolerated by the diplomonads than is the plasma of rainbow trout. Thus our results may indicate the following hierarchy of strength of plasma against *S. salmonis*: sturgeon > carp > rainbow trout.

Since *S. salmonis* infection in common carp has not been reported, I expected that the plasma from carp would consistently show stronger lytic affect than plasma from rainbow trout. Several reasons may explain the similar lytic effect of rainbow trout and carp plasma as follows:

(i) Rainbow trout which have been used in the present study were not naive fish (the fish had been exposed to *S. salmonis*). A more rigorous and appropriate comparison between species would be to use plasma from the fish that had never been exposed to *S. salmonis*. If this was not possible, a compromise would be to use heat inactivated plasma, in which the complement generated from both classical and alternative pathway would be inactivated, but antibody will be still active.

(ii) The data was obtained from only 3 rainbow trout, and 2 carp. It is indeed probable, that larger sample sizes would yield less variability in the data, and that the true differences between rainbow trout and carp would be clearer. In our studies, although many more fish were available, the small sample sizes in the experiment were due to limited availability of trophozoites in fish and in culture.

(iii) Rainbow trout is known to be a common host of *S. salmonis* (Ferguson 1979, Poynton et al. 2004), whereas common carp was reported to be a host for other *Spiornucleus* species (which were observed in the rectum of common carp in China, and named as *S. carassii*) (Wu et al. 2003). If further tests showed little differences between the plasma of rainbow trout and carp, this could be explained by the fact that the test reflects susceptibility to diplomonad infection at the level of genus of parasite, rather than species level.

(iv) It is possible that common carp is indeed susceptible to *S. salmonis*, even though this has not been reported in the literature. In the wild, carp and rainbow trout are not usually present in the same water body, so natural cross infection is unlikely. Furthermore, there does not appear to be any literature reported the experimental transmission of *S. salmonis* to common carp. Experimental infections could be undertaken to determine if *S. salmonis* can be transferred to, and established in, common carp. If establishment is confirmed, this may explain the small differences between carp and rainbow trout plasma in the present study.

5.5.7 Discussion: Compare *in vitro* plasma test with epizootiological data

Rainbow trout is considered to be a normal host for *S. salmonis* according to the epizootiological data (Ferguson 1979, Poynton et al. 2004). This is in consistent with the *in vitro* plasma incubation test which shows that *S. salmonis* trophozoites can remain alive in the rainbow trout plasma for over 120 min in the 1:64 plasma concentration, longer than that in carp and sturgeon plasma.

The diplomonads reported from rectum of common carp in China as *S. carassii* (Wu et al. 2003), and from gall bladder of 3 sturgeon species in Russia as *H. truttae* in (Bauer et al. 2002) have been studied by light microscopy, which is not reliable technique to distinguish diplomonad genera and species. Thus the susceptibility of carp and Atlantic sturgeon to *S. salmonis* is not known.

5.5.8 Further study

In the present study, if more diplomonads in culture were available, I would have pursued the between species tests with higher numbers of fish of the different species. I could also have used the modified *in vitro* plasma incubation test for rainbow trout plasma from fish with different densities of infection, i.e. to use the test for within species. It would be expected that the plasma from uninfected rainbow trout plasma has stronger lytic and cytotoxic affects than the plasma from lightly, moderately or heavily infected fish.

The *in vitro* plasma incubation test was described and used by Bower and Woo (1977) to study innate immunity due to the alternative complement pathway activation. The complements, which are generated via the alternative pathway, contribute to effectively eliminating the pathogens during the early stage of the infection. The heat-inactivated plasma was used to detect the complement in blood, which be activated via the alternative pathway (Forward and Woo 1996). In the present study, the limited availability of diplomonad cultures did not allow further studies beyond protocol development and demonstration, to allow me to investigate the use of heat-inactivated plasma (the plasma is incubated at 56 °C for 30 min). This needs to be done in further studies. If the fresh plasma in resistant hosts (carp and sturgeon) has lytic and cytotoxic affects, but the heat-inactivated plasma has not, then we can conclude that the resistant fish has heat labile elements (possibly from the alternate complement pathway) that are damaging to the parasite. This may indicate their innate immune response to *S. salmonis*.

CHAPTER 6

SYNTHESIS AND CRITIQUES

CHAPTER 6

Synthesis and critiques

6.1 Synthesis

The present study not only accomplished our understanding of *S. salmonis* as fish parasite as expected, but also gave enough information for as cross fertilization / translation research to other areas; i.e. new routs for management and treatment (anti mucus, and anti adhesion therapy), the potential for new lab model for study of flagella attachment and their fundamental biological process, or very significant outcomes of unplanned studies on trophozoites to cyst transformation in piscine diplomonad *Spironucleus*. In this chapter, I synthesized an overview of the information that has not been given in previous discussions, since most of those focused on contents of published or submitted papers; and criticized each of the five parts of the thesis (characterisation, intestinal pH and the microhabitat preference, multi-functionality of flagella, pathology, and *in vitro* plasma incubation test) concludes with an outlook and overview.

6.2 Critiques

6.2.1 Characterisation

This thesis has accomplished the first comprehensive study on *Spironucleus salmonis* in rainbow trout in Germany, and has completed the description of *S. salmonis* presented by Poynton et al. (2004) (which lacked detailed SEM and molecular characterisation). Taking samples from other parts of the Germany, and indeed other countries, may confirm *S. salmonis* from other rainbow trout farms, and show if there are different genotypes. Even in countries such as Denmark where pathogenic diplomonads are common in farmed trout,

there are few comprehensive characterisations of the parasites. It would be interesting and of practical value to know if the same genotype of *S. salmonis* is present in Germany and Denmark, and if it is different, this may explain why culture of the organisms from Germany was not very successful, even when using a protocol developed for "*H. salmonis*" in Denmark.

The novel aspect about the characterisation study was the understanding of the role of organelles based on their morphology - "functional morphology". For example, observing the vacuoles in the surface of the cell membrane that discharge their contents to outside; this has not been documented in detail before. Thus their interaction with the outside of the cell is not only from flagellar pocket as previously reported. The other interesting new observation of the function was the presence of lobes in the nuclei. This phenomenon had been observed in other *Spironucleus* species like *S. barkhanus*, *S. torosa*, and *S. vortens*, however, their possible function has never been discussed before. The lobes in the nuclei may indicate how the beating of flagella produces force and pressure to the kinetosome and the apex of the cell. This pressure can be accommodated by the occurrence of these lobes in the nuclei. It is apparent that the form of lobes is deepened according to the type and the severity of the flagella beating.

The ultrastructural study gave also insight into two possible new species-specific diagnostic tests. Although ultrastructural and molecular diagnostic methods are the most precise methods of recognition (Poynton and Sterud 2002), most fish health laboratories cannot afford to use ultrastructure for diagnostics (it is expensive and time consuming), and molecular methods are still in development. Therefore an alternative simple and cheap method for a quick way of distinguishing genus and species is essential.

In *S. salmonis*, a diagnostic test can be developed and used based on the 8-shaped sac of dense ribosome at the posterior end of the cell. PAS stain (periodic acid schiff), is the most regularly used staining technique for demonstrating glycogen which stains pink-red. This can be used to distinguish the uncoloured sac at the posterior end of the cell from red-coloured anterior and middle of cytoplasm under light microscope. This fast and easy

technique, carried out on smear preparations, will help diagnostic laboratories to distinguish *S. salmonis* from other *Spironucleus* species. This shows how morphology can have implications in identifications.

An additional kind of test can be developed because each diplomonad species has a unique cytoskeleton and suite of cytoplasmic organelles, some features of which were visualised in early light microscopy studies; others have only been recognised in recent electron microscopy studies. At present there is only one cytoskeleton stain, and no cytoplasmic stain for species-specific features of piscine diplomonads that can be observed by light microscopy. Protargol silver protein stain is used to distinguish *S. torosa*, *S. vortens* and a diplomonad tentatively identified as *H. salmonis*, based on differences in accessory cytoskeleton at the posterior end of the body (Poynton and Morrison 1990, Poynton et al. 1995). However, smaller elements cannot be resolved due to poor resolution; furthermore protargol is complicated, and time consuming.

Recently, immunofluorescence microscopy has been used to demonstrate tubulin (microtubules) diversity in *Giardia lamblia* trophozoites by Campanati et al. (2003). To determine how *Giardia* swim and divide, trophozoites have been labelled with an amino-specific Alexa Flour dye that highlighted the flagella and adhesive disc by Ghosh et al. (2001). However, no paper has been published on use of immunofluorescence microscopy in *Spironucleus* species, although it clearly holds great potential. Immunofluorescence can show the accessory cytoskeleton of diplomonads from rainbow trout. The further study on this issue can demonstrate the pattern of cytoskeleton and microtubular band of *S. salmonis* from German rainbow trout and other *Spironucleus* species from fish, using immunofluorescence microscope to see labelled α -tubulin.

Another important fact about my study was depositing SEM stubs, TEM block, and the sequence of *S. salmonis*, which can help other researches for their follow-up study.

Further aspect of this work was the establishing a collaboration study between my study in Germany and our colleagues in Norway. Although there was close collaboration, there was

no overlap and each team focused on different aspects of the work. I focused on identifying using ultrastructure, and they assisted in sequencing. Our team work provided a comprehensive study of this organism. This joined work still continues and is going to be extended to diagnosis of other species of diplomonads in other vertebrate hosts including birds.

6.2.2 Intestinal pH and the microhabitat preference

Understanding microhabitat preferences of *S. salmonis* in rainbow trout will open new insight into mechanisms of the interaction between this protozoan parasite and microenvironments of their host intestine. It is possible that many factors explain the preference of *S. salmonis* being enteric in pyloric region, however not all these factors were considered in my study. Therefore, a clear picture of the relationship between the parasite and the conditions in which it lived within the intestine is still needed.

The majority of the attention for this research was based on the pH factor (not as the only possible factor, but as one of the factors) in determining of their microhabitat preference, and this poses questions for further research, including research on the other environmental factors affecting *S. salmonis* like the amount of bile and the amount of nutrition in the environment (contents of intestine). The amount and type of carbohydrates, proteins and other factors that can be found in the contents of intestine creates a new dimension for determining a successful protocol for culturing this organism.

My study of pH *in vivo*, in the pyloric region, brought precision to the data suggested for optimal pH in the previous *in vitro* study (Buchmann and Uldal 1996). Optimal pH, in the culture, fluctuated in the range of 7.5 to 8.0 (Buchmann and Uldal 1996); in our natural study this optimal pH was precise to 7.3. This information is a significant aid to successful *in vitro* growth of this organism.

The practical impacts of my findings in intestinal pH is not only more efficient culturing of *S. salmonis*, but also predicting the likely impact of changes in diet (and consequent changes of

pH) on density of infection. My pH study creates room for discussion on the affect of diet on the relationship between parasite and host.

6.2.3 Multi-functionality of flagella

The novel observation on attachment, formation of cysts clusters, and the encystment process of *S. salmonis* was not originally included in my PhD project. However the unexpected chance to pursue this was most valuable; and the results help to explain the previously unknown mechanisms for formation of clusters of cysts in piscine diplomonads – a poorly known part of their life cycle. This observation brought our attention to a new role of flagella in *S. salmonis* and that was their ability to adhere. Besides multi-functionality of flagella, the role of the other organelles related to this adhesive ability appeared to be clearer now. For example, I am now speculating that the cell membrane has 5 different zones based on this observation, and the possible role of the flagellar pocket and the 8-shaped sac in flagellar adhesion is now considered.

A difficulty for my understanding and interpretation of this phenomenon creates two options; either what I have discovered, analysed and interpreted is consistent with what happens in practice (i.e in nature), or it is only an artefact.

One can speculate that the flagellar attachment happens (adhesion ability is there), but it is only possible for this adhesion to take place in the lab, not in the natural environment. The factor(s) in the culture which allow the trophozoites to be adhesive may never been available in the natural environment where the organism live. In the other words, the flagella have the ability to become an adhesive organelle, but this can only been recorded in laboratory conditions. The question that why the adhesion takes place in lab conditions is one that requires more research to be conducted.

Although repetition of culture confirmed our observation, this did not exceed more than 7 times, because of the failed attempts in culturing (only about 30% of cultured were successfully sub-cultured). It is normal to have cyst in culture because this produces

improper conditions for cells so they start creating cysts. In my experiment, only one of the two culture protocols provides cysts; however, the only difference between the two culture protocols was the type of serum. In the culture that produced cysts, the serum was newborn calf serum, and in the culture that produced trophozoites, the serum was bovine serum. The other materials and their doses were similar in both culture protocols. I was unable to show which exact factor was in the culture that initiated cyst production. However, this is very important issue to consider for further studies.

The interaction of trophozoites with each other or debris through adhesive posterior flagella could be confirmed by detecting adhesive molecules or proteins in the flagella. This did not happen, because of the time limitation, and lack of equipment and experience.

Even with considering all of these debates and discussions above, this observation of adhesion and attachment was very important and cannot be easily dismissed. This sort of behaviour (adhesion) is important and essential in the life cycles of other flagellates organisms such as kintoplastids or *Chlamydomonas*. So this can also be the case in the life cycle of *S. salmonis*. If this is true, then we can use this adhesion phenomenon for a practical application namely treatment. As mentioned in several parts of this dissertation, there are many difficulties when using chemical treatments against diplomonad infection. Therefore, if clustering is important for the life cycle or for initiating a new infection, there may be an alternative treatment that could be developed, targeting the cyst clusters instead of the trophozoites, and directed against the adhesion.

One difficulty in this study was to recognise the solitary cysts in the culture due to their lack of movement. A possible option can be adding stain to the culture that will be absorbed by the cyst wall. In order to reach to this point, several key factors need to be accomplished: (i) establish a successful and repeatable culture media that could produce large numbers of the cysts, (ii) obtain knowledge of the structure of the cyst wall in *S. salmonis*, and (iii) doing the experiments to identify the staining material which could influence the cell wall, and making the cyst visible. Once the cysts are recognisable in the culture, we can then use this method in vivo. This meant that for the first time, we can observe the cyst in its natural state as either a

solitary cell or in a cluster. This procedure will allow us to answer our unresolved question about whether the cluster was artificial, or if it also occurs in its natural environment.

6.2.4 Pathology

Intestinal diplomonad infections in rainbow trout are commonly associated with lack of appetite, and decrease weight gain. The mechanism was unknown. Rigorously controlled studies were needed, yet had not been done. The results presented in this thesis show that hypertrophy and hyperactivity of goblet cells is present in *S. salmonis* infected fish, and we suggest this may contribute to poor nutrient absorption, and this in turn may lead to decreased weight gain.

Preliminary report of Ferguson (1979) suggested decrease absorptive surface of intestine in heavily infected fish and excess mucus, yet lacked density data. In some of our TEM sections, we also noted cytoplasmic blebbing, with swelling and loss of microvilli at the apices of the epithelial cells. Therefore, demonstrating histopathological changes with differing density of diplomonads might reveal mechanisms of pathogenesis. I tried to address this lack of knowledge by developing a quantitative study, for the first time, to demonstrate whether *S. salmonis* infection is related to enteropathy.

I had originally planned a comprehensive study to compare the severity of the different pathology parameters in four densities of infected fish: uninfected, light, moderate and heavily infected fish, with samples of 25 fish per density. However, difficulties in obtaining sufficient numbers of infected fish have caused me to modify the study to comparison of two densities, uninfected and moderately infected, and change the sample size to 15 fish per density.

If I could study the possible relationship between density of infection and severity of enteropathy, and if significant differences in pathology with density of infection were found, this may indicate causality and suggest a possible mechanism by which the parasite causes the clinical signs. However, the modified study was still a very valuable investigation, since

it established a new quantitative protocol. Meanwhile, the present quantitative pathology study shows that this “quantitative” study can be expanded and performed, when the infection is available, to find the relationship between density of infection and severity of entropathy.

6.2.5 *In vitro* plasma incubation test

I focussed on detecting the presence of the innate immunity mechanism (the alternative complement pathway) to *S. salmonis* in the plasma of resistant fish by modifying *in vitro* plasma incubation test. I successfully modified the plasma incubation test, previously used for hemoflagellates (Bower and Woo 1977), and used it to detect lysis and cytotoxicity of the enteric flagellate *S. salmonis*.

The reduced infection resources (see section 6.3 below) did not yet allow me to collect sufficient data to complete this study. However, the plasma incubation test protocol has been established, and appears to predict the potential susceptibility of different species of fish to *S. salmonis*. This would be a valuable tool in management of infection.

During my experiment on the effect of susceptible and resistant fish plasma on *S. salmonis*, I noticed similarities between plasma of rainbow trout (susceptible) and carp (resistant). It is essential in further studies to create an experimental infection in carp to observe whether the fish can carry the infection of *S. salmonis*.

It is also of interest to me to discover the affect of plasma of rainbow trout infected with varied density of infection (light, moderate, and heavy) on *S. salmonis* (in vitro plasma incubation test within species).

Decreasing prevalence of diplomonads with age in rainbow trout (Uldal and Buchmann 1996) may indicate that fish become immune after exposure as fry and fingerlings. Although there are no reports of immunodiagnosis of any *Spironucleus* species in fish, based on what is known for *Giardia*, we would expect testing for the presence of serum antibodies to

Spironucleus via indirect fluorescent antibody (IFA) technique to be effective; we did not study this, because of time and equipment limitations.

6.3 A note on limited availability of infected fish

During the last years of my Ph.D. research, there were frequent difficulties to get enough infected fish for collection of heavily infected fish to inoculate cultures, and from which to collect pathology samples, (i.e. I examined 4 times more fishes than anticipated in order to get enough pathology samples for the research). The infection prevalence at our usual resource farm, an open pond farm near Berlin, decreased significantly, and remained low, most infections were light, and some were moderate. The lower infection in the farm was probably related to the unusual warm weather, especially in summer 2006, which was the warmest and longest in the last decade. *Spironucleus salmonis* is very temperature sensitive, in vitro studies show that the number of parasite declines significantly at temperatures > 15 - 20 °C (Buchmann and Uldal 1996). A further complication is that a stock source of *S. salmonis* in culture was not available from ATCC (American Type Culture Collection), or from any other known source.

6.4 Overview and outlook

The research yielded significant results, some of which were anticipated, others not. My research will not only support improved diagnosis, management and treatment of *S. salmonis* infection, but also has important applications to other fields of biology.

In summary:

The anticipated results that were achieved during this Ph.D. research were:

- **Characterisation**
 - Ultrastructure and molecular characterisation
 - Functional morphology of *S. salmonis*
 - Should allow for better diagnosis, epizootiology
- **Microhabitat preference**
 - pH is shown not to be a major determining factor
 - Should allow more efficient *in vitro* culture
 - May help to predict effect of changes in diet
- **Pathology**
 - First quantitative study of enteropathy and infection
 - First report of hypertrophy of goblet cells
 - New treatment target using anti mucus
- **Innate immunity**
 - Modify in vitro plasma incubation test for diplomonad
 - Predict host susceptibility to *S. salmonis*

The unexpected results and their future applications were:

- **Multi-functionality of flagella**
 - Cells form clusters, initiated by adhesive posterior flagella
 - First demonstration of multi-functionality of flagella in diplomonads,
 - Suggests five functional domains of the cell surface
 - Indicates new treatment target using anti-adhesion therapy

New diagnostic tools – identification of techniques and targets PAS, cytoskeleton

Better conditions for *in vitro* culture – more accurate documentation of optimal pH

Identification of possible new treatments – decreased mucus production,
anti-adhesion therapy

New aspect of link in the life cycle – transformation of trophozoites to clusters of cysts
via adhesion

New model system for multi-functionality of flagella – heat stressed diplomonad,
culture, trophozoites to cysts

CHAPTER 7

LITERATURES

CHAPTER 7

Literatures

A

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